CHAPTER ONE

Pathogen and host genetics underpinning cryptococcal disease

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Contents

Abstract

Cryptococcosis is a severe fungal disease causing 220,000 cases of cryptococcal meningitis yearly. The etiological agents of cryptococcosis are taxonomically grouped into at least two species complexes belonging to the genus *Cryptococcus*. All of these yeasts are environmentally ubiquitous fungi (often found in soil, leaves and decaying wood, tree hollows, and associated with bird feces especially pigeon guano). Infection in a range of animals including humans begins following inhalation of spores or aerosolized yeasts. Recent advances provide fundamental insights into the factors from both the pathogen and its hosts which influence pathogenesis and disease. The complex interactions leading to disease in mammalian hosts have also updated from the availability of better genomic tools and datasets. In this review, we discuss recent genetic research on *Cryptococcus*, covering the epidemiology, ecology, and

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Advances in Genetics, Volume 105
ISSN 0065-2660
https://doi.org/10.1016/bs.adgen.2020.02.001

evolution of *Cryptococcus* pathogenic species. We also discuss the insights into the host immune response obtained from the latest genetic modified host models as well as insights from monogenic disorders in humans. Finally we highlight outstanding questions that can be answered in the near future using bioinformatics and genomic tools.

1. Introduction

Cryptococcosis is a severe fungal infection of primarily the central nervous system and lungs of a variety of animals including humans (Rajasingham et al., 2017). The etiological agents of cryptococcosis are taxonomically grouped into at least two species complexes of the genus *Cryptococcus* (*C. neoformans* and *C. gattii*). All of these yeasts are environmentally ubiquitous fungi (often found in soil, leaves and decaying wood, tree hollows, and associated with bird feces especially pigeon guano). They also possess a polysaccharide outer layer (capsule), which is a unique feature in members of the fungal kingdom (Zaragoza, 2019). Infection in a range of animals including humans begins following inhalation of spores or aerosolized yeast or hyphae.

The first description of cryptococcosis occurred in the mid-1890s from a patient with chronic periostitis of their tibia. Independently the yeasts were observed within fermenting peach juice (Barnett, 2010; Busse, 1894). Recent estimates indicate approximately 220,000 cases of cryptococcal meningitis occur yearly, particularly in those living with HIV/AIDS (Rajasingham et al., 2017). Nearly ¾ of cryptococcosis cases are from sub-Saharan Africa, while the remaining cases are mostly from Asia and the Pacific (Rajasingham et al., 2017), reflecting their HIV/AIDS burden. Access to highly active anti-retroviral therapy has dramatically reduced AIDS and AIDS-related cryptococcosis. However any individual with an immunosuppression, such as the iatrogenic immunosuppression of transplant patients and autoimmune disease patients, as well as other conditions, such as chronic liver pathologies, are at increased risk for cryptococcal disease. For example, cryptococcosis can affect up to 20% of solid organ transplant recipients in the United States (Singh et al., 2008). *Cryptococcus* will therefore remain a challenge for global health for the foreseeable future.

In this review, we discuss recent genetic research on *Cryptococcus* and cryptococcosis. One of our main foci is on the epidemiology, ecology,

and evolution of *Cryptococcus* pathogenic species, all of which have been recently revised. Knowledge of the complex interactions leading to disease in mammalian hosts has also benefited from the availability of better genomic tools and datasets. We describe recent advances that have started giving fundamental insights into the basis of pathogenesis in both the pathogen and its hosts. We also highlight important outstanding questions that we anticipate will be answered using state-of-the-art bioinformatic and human population genetic studies.

2. *Cryptococcus* spp. genetics and genomics

2.1 The *Cryptococcus* genus

The *Cryptococcus* genus is comprised of around 40 described basidiomycetous species and species complexes (comprising several species or lineages). Prior to 2011, *Cryptococcus* referred to the asexual/anamorphic state only (yeast-like), while the sexual/teleomorphic state (filamentous) of the *Cryptococcus* genus was named as *Filobasidiella*. However, in July 2011, at the XVIIIth International Botanical Congress in Melbourne, Australia, the Nomenclature Section of the Congress adopted the resolution from Hawksworth (2012) to have "one fungus, one name" (Taylor, 2011), and now *Cryptococcus* is used for all species in the genus, regardless of anamorphic or teleomorphic states.

Our focus in this review is the pathogenic species encompassed by the two species complexes *Cryptococcus neoformans* and *Cryptococcus gattii*. The morphologies of both are dynamic (Fig. 1), with various stimuli and environments shifting it away from its usually studied yeast phase (Fig. 1A). Albeit in comparison to *A. fumigatus* and *C. albicans* who form aggressively invasive hyphae during infection, *Cryptococcus* is primarily a yeast during mammalian infection and routine laboratory conditions. However, *Cryptococcus* can grow as hyphae during mating (Fig. 1B and C), and as pseudohyphae upon contact with predatory amoeba (Fig. 1D). During mammalian infection, *Cryptococcus* also enlarges its capsule outer layer (Fig. 1E), and forms dramatically enlarged cells named "Titan cells" (Fig. 1F). The other yeasts of this genus are less studied, and the full repertoire of morphologies are less known, although some relatives such as *C. humicola* appear to also have a hyphal to pseudohyphal forms in laboratory conditions (Kwon-Chung, Boekhout, Wickes, & Fell, 2011).

All *Cryptococcus* spp. belong to the Tremellaceae family within the Tremellales order. Tremellales include a number of human pathogens other

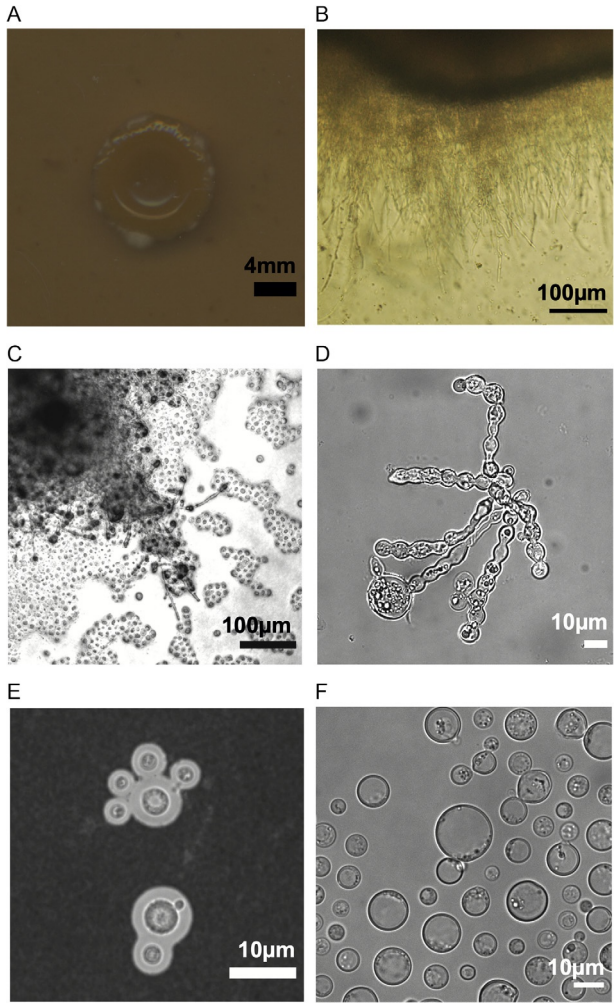

Fig. 1 Morphology of *Cryptococcus*. (A) Colony morphology after dikaryon mating in V8 agar reprinted from Maryam et al. (2019). (B) Visible hyphae forming at the edge of the fungal colony. (C) Morphology after predation by amoeba: hyphae are induced after exposure to predation by the amoeba *Acanthamoeba castellanii*. (D) Pseudohyphal forms are also observed as these yeasts are predated. (E) Morphology after infection of mammalian host: capsule is induced, as shown by negative staining with India ink. (F) *Cryptococcus* exposed for 24 h to murine macrophages, and then macrophages were lysed and capsule observed. Titan cells (induced using the conditions described in Hommel et al., 2018; Maryam et al., 2019) an enlarged cell type which was first observed during infection of mammalian hosts. All images using *C. neoformans* H99. *Images produced by Carolina Coelho, with the help of Man Shun Fu and Alexandre Alanio.*

than the *Cryptococcus* species complexes *C. neoformans* and *C. gattii*, such as several dermatophytes from the *Trichosporon* genus, including *T. asahii*, *T. asteroides*, and *T. dermatis*. Tremellaceae also includes mycophages or parasites of other fungi, including the edible golden jelly fungus/witch's butter (*Tremella mesenterica*) that is found on all continents except Antarctica, typically on fallen branches of angiosperms where it is a parasite of wood-decay fungi in the genus *Peniophora* (Zugmaier, Bauer, & Oberwinkler, 1994). Tremellaceae were originally categorized based on having gelatinous fruiting bodies in 1821 (Fries, 1821), and re-categorized in 1900 for having "tremelloid" basidia, i.e., spherical or ellipsoid spore-bearing structures with vertical or diagonal septa that produce basidiospores (Patouillard, 1900). However, it was later shown that neither classification was accurate, given that species belonging to the *Cryptococcus* genus that phylogenetically group within the Tremellaceae family do not have tremelloid basidia, which are instead globular at the apex and cylindrical at the base (Velagapudi, Hsueh, Geunes-Boyer, Wright, & Heitman, 2009). Therefore, the best classification of the genus is based on the genetic distance between species rather than morphology.

The closest relatives of *C. neoformans* and *C. gattii* are the non-pathogenic species *C. amylolentus* and *C. wingfieldii* (previous *Tsuchiyaea wingfieldii*) (Findley et al., 2009). *Cryptococcus depauperata* (formerly *Filobasidiella depauperata*) is only known to grow as hyphae found in association with decaying insects (Rodriguez-Carres, Findley, Sun, Dietrich, & Heitman, 2010), and is another close relative of the *neoformans/gattii* species complex. Together, these species form a group described as the *Cryptococcus* sensu stricto group (Findley et al., 2009). At least five other species (*Bullera dendrophila*, *Kwoniella mangroviensis*, *Cryptococcus bestiolae*, *Cryptococcus dejecticola*, and *Cryptococcus heveanensis*) form the Kwoniella clade, a sister group to the *Cryptococcus* sensu stricto group (Findley et al., 2009). Kwoniella is ecologically and geographically diverse, and includes yeasts that are saprobic, insect-associated/found in insect frass, found on sheet rubber in Indonesia (e.g., *C. heveanensis*), and in mangrove habitats in the Florida Everglades as well as the Bahamas (Statzell-Tallman, Belloch, & Fell, 2008).

A distinguishing feature for all *Cryptococcus* species is the polysaccharide capsule that encapsulates the yeast form, which is a very unique structure for the fungal kingdom. The capsule is comprised of glucuronoxylomannan (GXM), galactoxylomannan (GalXM) and mannoproteins. GXM structure differences determined serotypes which were used as differential classification of strains (Zaragoza et al., 2009). Related Tremella species including

the *T. mesenterica*, silver ear/snow fungus (*T. fuciformis*) and golden ear (*T. aurantia*) are also able to produce extracellular polysaccharides, although their composition appears to be different to that of *Cryptococcus* (De Baets & Vandamme, 2001). Curiously, in all of these species these polysaccharide structures provide some degree of protection to the yeast but are being harnessed for their immunomodulatory properties (Vecchiarelli et al., 2013). Indeed, the capsule and polysaccharides of these species are studied for a range of potential protective or pro-immuno-modulatory effects including antitumor, anti-oxidation, anti-aging, hypoglycemic, hypolipidemic, and neuroprotection (Decote-Ricardo et al., 2019; Yang, Liu, & Zhang, 2019). Differences in physiological responses may be caused by the chemical differences between species respective polysaccharides. For example, *Cryptococcus* has a higher proportion of mannose and glucuronic acid, and fewer xylose residues linked to the mannose backbone of the polysaccharide, although the functional importance of that remains to be determined.

C. humicolus is another rare opportunistic pathogen closely related to *C. curvatus* and *Trichosporon* species based on ITS and 18S rDNA based phylogenetics (Sugita, Takashima, Ikeda, Nakase, & Shinoda, 2000). Little is known about *C. humicolus*, although it was found to be the cause of a single case of disseminated systemic cryptococcosis in a 7.5-year-old HIV-negative boy in Rasta Peth, Pune, India in 2004, and cured after successful antifungal treatment (Shinde, Vanarse, & Pandit, 2004). A second isolated case of *C. humicolus* was reported in Kuala Lumpur, Malaysia in 2010 when a 49-year-old immunocompetent patient with meningitis symptoms tested positive using a cryptococcal antigen test on CSF and *C. humicolus* was subsequently cultured (Ramli et al., 2012). The distribution of *C. humicolus* and its normal niche in the environment or in other hosts is unclear. However its ability to cause disease in apparently immunocompetent humans suggests further research into this particular species is needed.

C. uniguttulatus has been isolated from droppings and cloacal swabs of feral pigeons (*Columba livia*) in Malmö, Sweden (Mattsson, Haemig, & Olsen, 1999) along with isolates of the suspected saprobe and rare human pathogen *Cryptococcus laurentii*, and the distantly related ascomycetous yeast *Debaryomyces hansenii*. *C. uniguttulatus* has also been found as a contaminant of laboratory rodent chow, while *C. laurentii* was found in air samples at the School of Veterinary Medicine in Louisiana, USA (Mayeux, Dupepe, Dunn, Balsamo, & Domer, 1995). *C. uniguttulatus* is unable to grow at

37 °C, and is more closely related to *C. capsuligenum* (formerly *Filobasidium capsuligenum*) than *C. neoformans* (Kurtzman, Fell, & Boekhout, 2011). Despite not growing at 37 °C, it has been isolated from nail infections and even a case of ventriculitis, demonstrating that it is capable of causing disease (Kurtzman et al., 2011).

An unusual *Cryptococcus* species is *C. curvatus*: an extremophile found in cold seep/cold vent sites where hydrogen sulfide, methane and other hydrocarbon-rich fluid escapes from the ocean floor. For example, studies on the cold-seep sediment of Sagami Bay and Kuroshima Knoll located in the Southern Ryukyu Arc near Ishigaki Island, Japan, showed *C. curvatus* overwhelmingly dominates (71%) the microeukaryote communities of both cold-seep sites (Takishita, Kakizoe, Yoshida, & Maruyama, 2010). In those sites, *C. curvatus* were recovered from sediments which also contained a diverse range of ciliates that are likely bacterivorous on archaea and bacteria oxidizing sulfides and methane in that ecosystem. While the exact niche of *C. curvatus* and ecological associations with ciliates are unclear, *C. curvatus* is noted for being oleaginous—utilizing and breaking down lipids, and producing "hairy and warty protuberances"—outgrowths of the cell walls following growth on high levels of polymerized triglycerides (Leeuw et al., 2010).

Genomic studies have demonstrated a huge phylogenetic diversity within the *Cryptococcus* genus. Several separate pathogenic species have been described so far, with a handful of additional species also being rarely associated with human disease, as detailed in this chapter. All pathogenic species of *Cryptococcus* frequently target the lung and the brains of mammals, and their antifungal drug susceptibility profile is not restricted to species boundaries (Chen, Meyer, & Sorrell, 2014; Hagen et al., 2017; Kwon-Chung et al., 2017). For example, while the majority of studies refer to *C. neoformans* (usually encompassing *C. neoformans* (var. *grubii*) and *C. neoformans* var. *neoformans*/*C. deneoformans*/VNIV), several lineages of *C. gattii* were shown to cause fungal meningitis and disseminated infection (Walraven et al., 2011). *C. gattii* is also a very prevalent cause of HIV-associated deaths. Autopsy studies showed *C. gattii* to be the responsible for up to 30% of HIV-associated cryptococcosis deaths in Brazil and Mozambique (Hurtado et al., 2019), and *C. gattii* are also highly prevalent in California (Springer et al., 2014).

One particular outbreak of cryptococcosis that is particularly noteworthy in terms of number of cases is the Vancouver/British Columbia outbreak of

C. gattii by a number of clonal subclades of *C. gattii* including VGIIa (e.g., isolate R265). R265 is particularly virulent strain causing infections in apparently immunocompetent individuals. Because this outbreak and other *C. gattii* infections are often characterized by pneumonia in immunocompetent individuals, it suggests a preference of *C. gattii* for causing lung disease. However this has not been widely shown and *C. gattii* isolates can be responsible for HIV-associated meningitis (Hurtado et al., 2019; Springer et al., 2014). There are also examples of immunocompetent individuals presenting with meningitis caused by *C. gattii* (Amburgy et al., 2016), suggesting that the R265 lung tropism may not be truly representative of *C. gattii* infections. It also remains to be determined which pathogen or host factors are responsible for the striking neurotropism of *Cryptococcus*.

The difficulty in separating disease caused by the two pathogenic *Cryptococcus* species complexes into different clinical entities is reflected in laboratory immunological studies. While different strains of *C. gattii* show different profiles in capacity to elicit cytokine production from human cells (Herkert et al., 2018), no specific link between genotype at the species level and virulence was found yet (Firacative et al., 2016; Kwon-Chung et al., 2017). Solving this conundrum would require genotyping isolates and associated clinical manifestations in a large number of patients. Overall we support the view posited by Kwon-Chung et al. (2017): thus far *Cryptococcus* pathogenic yeast species are indistinguishable clinically, and in the absence of species-level phenotypes, then disease should be noted as caused by pathogenic *Cryptococcus* or one of the two pathogenic *Cryptococcus* species complex. These discussions highlight that no distinction has been firmly established (we know we don't know), and is not an affirmation that distinction does not exist. Thus, for the purpose of this review, we will refer to the *Cryptococcus* species complexes as simply *Cryptococcus*, and only differentiate species and/or lineages when required. Furthermore, the lineage names currently used to distinguish lineages within these two species complexes serve as a practical "zip-code," offering a convenient way to describe newly discovered lineages or recombinants from across the complexes.

Many questions remain about the evolution and ecology of the *Cryptococcus* genus that await a thorough systematic study. For example, some isolates of the basidiomycetous yeast *Filobasidium capsuligenum* produce a killer toxin (FC-1) against *C. neoformans*, *C. gattii* and *C. laurentii*, while other *Cryptococcus* species such as *C. albidus* and various other phylogenetically diverse species (*C. bisporidii*, *R. mucilaginosa*, *Candida* spp., *Debaryomyces* spp., *Kluyveromyces* spp., *Pichia* spp. and *Saccharomyces* spp.) were not sensitive

to the toxin (Keszthelyi, Hamari, Pfeiffer, Vágvölgyi, & Kucsera, 2008). FC-1 was proposed to bind β-1,6-glucan, a common component of the cell wall of fungi. However, the toxin specificity found may hint at a co-evolution of certain pathogenic *Cryptococcus* species with *Filobasidium capsuligenum*, which has also been isolated from multiple continents and from a variety of sources (brewery, soil and fruits) (Keszthelyi et al., 2008).

Comparing the genomes of the main pathogenic *Cryptococcus* species (*C. gattii* and *C. neoformans*) to other *Cryptococcus* and even Tremellaceae spp. that are considered entirely or largely non-pathogenic/saprophytic or pathogenic of different hosts has revealed novel genes or gene expansions involved in the transition to pathogenicity, and provide further information pertaining to its evolution, ecology or epidemiology. One notable recent study compared full-length chromosome assemblies between *C. neoformans*, and its closest non-pathogenic relatives in the *Cryptococcus* sensu stricto group (Findley et al., 2009): *C. amylolentus*, *C. wingfieldii* and *C. floricola* (Passer et al., 2019). The authors found approximately 6% pairwise sequence divergence between those species, accompanied with significant genomic changes, including inversions as well as a reciprocal translocation that involved inter-centromeric ectopic recombination (Passer et al., 2019), which likely impose significant barriers to genetic exchange. However, the gene content differences of these isolates, or gene divergence of certain pathways were not described—some of which may be important in the evolution of pathogenicity within the *Cryptococcus* genus.

2.2 Genetically distinct populations of cryptococcosis-causing *Cryptococcus* spp.

2.2.1 *Serotypes of* Cryptococcus

Heterogeneity among cryptococcosis-causing yeast isolates has been apparent from the middle of the 20th century with the recognition of four serotypes (A, B, C, D) based on the antigenic determinant of capsular polysaccharide (Evans, 1950; Wilson, Bennett, & Bailey, 1968). Serotype A (*Cryptococcus neoformans* var. *grubii*) includes the widely studied H99 isolate, which was the first *Cryptococcus* isolate to have its genome sequenced (Janbon et al., 2014), achieved via a collaboration between the fungal genome initiative at the Broad Institute of MIT and Harvard and Fred Dietrich at the Duke Center for genome technology. Isolate H99 derives from one of the first clinical isolates (H99O) which had lost virulence attributes due to multiple rounds of laboratory passages, was subsequently

passaged through a rabbit model of infection to regain virulence and subsequently distributed to various laboratories across the world (Janbon et al., 2014).

Serotypes B and C comprise isolates belonging to the *Cryptococcus gattii* species complex (Kwon-Chung, Boekhout, Fell, & Diaz, 2002). The majority of *C. gattii* have the serotype B capsular reactivity. Neither B nor C serotypes reflecting any true phylogenetic relationships between isolates. Serotype D (*Cryptococcus neoformans* var. *neoformans*/*C. deneoformans*/VNIV) is more commonly found in patients and in the environment from temperate countries such as Northern European countries. These isolates are more susceptible to heat killing (>47 °C) than serotype A strains, and display dermatotropism (Martinez, Garcia-Rivera, & Casadevall, 2001). Based on distinct sexual cycles and phylogenetic analysis using various gene sequences (Diaz, Boekhout, Kiesling, & Fell, 2005; Franzot, Salkin, & Casadevall, 1999; Xu, Vilgalys, & Mitchell, 2000), it was concluded that serotypes A and D belong to a single species complex named *C. neoformans* (Kwon-Chung & Varma, 2006) (Fig. 2).

In addition to the four serotypes belonging to the two species complexes, serotype AD hybrids are commonly found in both clinical and environmental samples from Sub-Saharan Africa where *C. neoformans* is thought to have originated (Litvintseva, Lin, Templeton, Heitman, & Mitchell, 2007). AD hybrids are also diploid or aneuploid in contrast to the widely common haploid *C. neoformans* isolates with either A or D serotypes. AD hybrids are more resistant to UV irradiation, at least compared to haploid serotype A strains from Botswana (Litvintseva et al., 2007). Hybrids between *C. gattii* and *C. neoformans* (e.g., serotype BD hybrids) are also rarely identified (Bovers et al., 2006), demonstrating that the species complexes are not entirely genetically isolated. Serotype BD isolates have been found from clinical samples and can be either diploid or aneuploid (Bovers et al., 2006).

Serotyping provided the first signs that the species is composed of genetically diverse populations of pathogens (Evans, 1950; Wilson et al., 1968). To date, a number of fundamental questions remain unanswered about *Cryptococcus'* serotype, including what the serotype specific epitopes are, the distribution of serotypes among lineages (e.g., both VGIII and VGIV includes serotype B and serotype C isolates), and if the serotype reflects a biologically meaningful trait that might be associated with virulence (i.e., a comparative virulence study). Serotyping can be

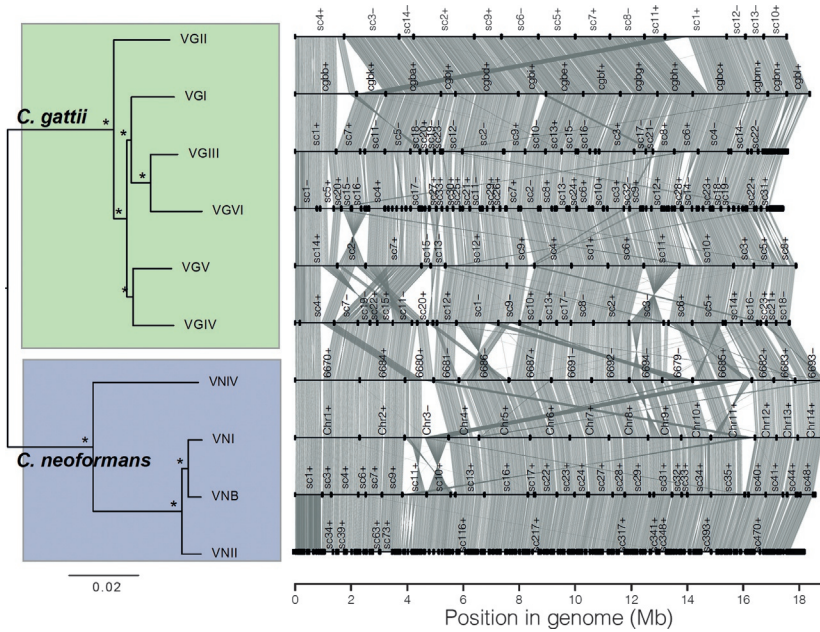

Fig. 2 (Left) A phylogenetic tree constructed of all known non-hybrid lineages of *C. gattii* (VGI → VGVI) and *C. neoformans* (VNI, VNII, VNIV and VNB) in RAxML v7.7.8 (CAT model of rate heterogeneity, WAG substitution model) (Stamatakis, 2006) based on multiple alignment of 3146 ortholog protein sequences identified using Orthofinder v2.2.7 (Emms & Kelly, 2015) through the Synima pipeline (Farrer, 2017). (Right) A syntenic representation of reference genomes belonging to each lineage produced by Synima (Farrer, 2017). VGI = WM276 (D'Souza et al., 2011), VGII = R265 (D'Souza et al., 2011), VGIII = CA1280 (Farrer et al., 2015), VGIV = IND107 (Farrer et al., 2015), VGV = MF34 (Farrer et al., 2019), VGVI = WM1802 (Farrer et al., 2019), VNI = H99 (Loftus et al., 2005), VNII = C45 (Rhodes, Desjardins, et al., 2017), VNB = Tu401 (Rhodes, Desjardins, et al., 2017), VNIV = JEC21 (Loftus et al., 2005).

achieved via a variety of agglutination tests with either antiserum or on immunofluorescence assays using a monoclonal antibody directed against the capsule polysaccharide (Enache-Angoulvant et al., 2007). The widely used "Crypto Check" kit (Iatron Laboratories, Tokyo, Japan) that was used since the mid-1980s was discontinued in 2004. However, subsequent PCR-restriction based methods and length polymorphism methods have since been used to determine serotype and may represent effective replacements (Enache-Angoulvant et al., 2007; Ito-Kuwa, Nakamura, Aoki, & Vidotto, 2007).

2.2.2 *The genetically distinct populations of* C. neoformans *(VNI, VNII, VNIII, VNIV, VNB)*

2.2.2.1 *C. neoformans* var. *neoformans*

The first *Cryptococcus* genomes sequenced were the VNIV (*C. neoformans* var. *neoformans*/*C. deneoformans*/serotype D) isolates JEC21 and B-3501A (Loftus et al., 2005), and the VNI (*C. neoformans* var. *grubii*/serotype A) isolate H99 (Janbon et al., 2014). Genomic analyses provided a rich resource for further population analyses and evidence of genomic architecture that hinted at karyotype instability and phenotypic variation. Both VNIV JEC21 and VNI H99 had genomes that spanned ~19 Mb across 14 chromosomes and encode ~6500 intron-rich genes (Loftus et al., 2005) (Fig. 2). A comparison between H99 and JEC21 showed that the two genomes are in overall synteny with only few chromosomal arrangements (Janbon et al., 2014) (Fig. 2).

Unlike *C. gattii* and *C. neoformans* var. *grubii*, sequencing and other genotyping methods for *C. neoformans* var. *neoformans* have to date yielded only the single lineage/species and which is known as a variety of names including clade I, AFLP1B and VNIV (Hagen et al., 2015) (Fig. 2). Phylogenetic placement of this lineage demonstrates it is more closely related to *C. neoformans* var. *grubii* than *C. gattii* (Fig. 2). VNIV has also been found to hybridize with *C. neoformans* var. *grubii* resulting in the hybrid molecular type/lineage AFLP3/VNIII (Hagen et al., 2015). Therefore, VNIV plays an important role in genetic exchange with other lineages, as well as persisting independently in the environment.

VNIII (serotype AD hybrids) are usually diploid (and therefore have two copies of the mating loci) or exhibit aneuploidy, show increased resistance to UV irradiation (Litvintseva et al., 2007), and are relatively common in environmental and clinical samples. For example, AD hybrids have been identified from China, Italy, Kuwait, and the United States (Litvintseva et al., 2007). In North Carolina, USA, 54/650 (7.1%) environmental *C. neoformans* isolates and 2.4% of clinical cases were serotype AD hybrids (Litvintseva, Kestenbaum, Vilgalys, & Mitchell, 2005). In a recent study, seven VNI/VNII (αAAα) and six VNII/VNIV (aADα) were studied for a range of phenotypes, finding that the VNI/VGII hybrid strains were more virulent in *Galleria mellonella* (wax moth) than the VNI/VGI strain (Aminnejad et al., 2012). Hybrids recovered from larvae also manifested an increase in capsule and total cell size and produced a low proportion (5–10%) of giant cells compared with the haploid control strains (Aminnejad et al., 2012).

2.2.2.2 *C. neoformans* var. *grubii*

C. neoformans var. *grubii* (serotype A) has been divided into at least three lineages: VNI, VNII and VNB (B for Botswana) (Litvintseva, Thakur, Vilgalys, & Mitchell, 2006; Meyer et al., 2009, 1999; Rhodes, Desjardins, et al., 2017). VNI and VNII are widespread, dominated by isolates with the MATα mating type, and predominantly clonal (Rhodes, Desjardins, et al., 2017). VNI is associated with trees and pigeon guano (Litvintseva et al., 2011), whereas the environmental reservoir for VNII is not well defined, and shows up primarily in clinical cases (Desjardins et al., 2017). Bisexual mating occurs in each of the lineages of *C. neoformans* between cells of complimentary mating types (MATa/MATα), i.e., heterothallic mating (Kwon-Chung, 1975), as well as unisexual mating between cells of the same mating types (MATα/MATα), i.e., homothallic mating (Lin, Hull, & Heitman, 2005). MATa/MATa diploids are also been reported, although it is unclear if these can undergo meiosis, perhaps owing to a yet to be identified meiotic inhibitor (Lin et al., 2005).

Meiotic recombination between either unisexual or bisexual reproduction results in similar reported counts of crossovers, recombination frequencies, and genomic hot and cold spots for recombination (Sun, Billmyre, Mieczkowski, & Heitman, 2014). *C. neoformans*, in contrast to *C. gattii*, can complete its life cycle (i.e., mating) on pigeon guano, where it is commonly identified (Nielsen, Obaldia, & Heitman, 2007), suggesting *C. neoformans* has adapted to mate on this substrate, although a mechanism for this predilection has not been identified. Indeed, close to 8% of natural *C. neoformans* isolates from across the world (mainly Asia, Africa, South America, and Australia) are diploid, which include a number resulting from same-sex (MATα/MATα) mating. However, the majority were auto-diploids comprising two identical copies of the genome that arose via either endoreplication or clonal mating (Lin et al., 2009).

The VNI global lineage is the most geographically diverse, whereas VNII is represented by a smaller number of locations including Africa, the Caribbean, Asia and North America (Rhodes, Desjardins, et al., 2017). VNI is comprised of at least three clades (VNIa, VNIb, and VNIc), which differ in their genetics, their geographic distributions, and their virulence (Beale et al., 2015). For example, isolates from the three clades had different cerebrospinal fluid (CSF) survival slopes (growth rates in pooled non-infected human CSF at 37 °C for 96 h, defined as <5 white blood cells per μL (Martín-Ancel et al., 2006)), with from low to high survival: VNIb, VNIc, and VNIa (Beale et al., 2015). While all three clades

were not clustered geographically in Southern Africa (Beale et al., 2015), reports from other regions such as in the Republic of Korea had as many as 93% of a single genotype: VNIc (Choi et al., 2010). Indeed, a recent study by Ashton et al. focusing on Asia and African *C. neoformans* found VNIa in 14 countries and 5 continents, VNIb in 10 countries in 6 continents, and VNIc in only 2 further countries: South Africa, and Botswana where it was however the most common clade ($n = 74/102$) (Ashton et al., 2019; Desjardins et al., 2017). In addition to being the most prevalent, the VNIa clade contains the highest genetic diversity ($\pi = 0.00178$) compared to VNIc (0.00125) and VNIb (0.00084) (Desjardins et al., 2017).

It is currently unknown what genetic differences might attribute phenotypic or geographical differences between the VNI clades, although major facilitator superfamily (MFS) transporters, and in particular the sugar transporter subset, were under positive or diversifying selection in all lineages of *C. neoformans* (Desjardins et al., 2017), perhaps indicating such changes may also be occurring between subclades and might make good candidates for their phenotypic differences.

Genome sequencing was recently used to compare 699 *C. neoformans* isolates from global sources, with a focus on Asia and Africa (Ashton et al., 2019). The newly sequenced isolates predominantly belong to VNIa ($n = 668$; 97.9%), with few VNIb ($n = 10$; 1/5%) and no VNIc. Ashton et al. found that just three subclades of the *C. neoformans* VNIa lineage were responsible for 91% of clinical isolates across five countries in Asia and Africa (VNIa-4 $n = 278/762$, VNIa-5 $n = 163/762$, and VNIa-93 $n = 143/762$). VNIa-93 was the most common subclade in Uganda and Malawi, and was linked to decreased mortality outcomes than either of the other two subclades, which predominate in Southeast Asia (Ashton et al., 2019). Further descriptions of the genetic differences between these three common subclades and other rarer isolates might indicate if there is a genetic component to their ecological or pathogenicity potential.

VNB has higher genetic diversity between isolates compared with VNI or VNII, has a greater mix of both MAT loci, and phylogenetic trees have longer tip branches for VNB isolates compared to VNI or VNII (Desjardins et al., 2017). Isolates belonging to VNB were originally identified in (and named after) Botswana. However, VNB isolates have since been identified from clinical isolates in Brazil and South Africa (Rhodes, Desjardins, et al., 2017). VNB include a significant proportion of fertile strains with the MATa mating type, and manifest compelling evidence of recombination

(Litvintseva et al., 2006). VNB has recently been shown to be deeply split into two genetically isolated lineages, VNBI and VNBII (Desjardins et al., 2017). The split of VNBI and VNBII also has some phenotypic disparity. For example, VNBII was enriched for clinical samples relative to VNBI, while VNBI isolates were more resistant to oxidative stress and more heavily melanized than VNBII isolates (Desjardins et al., 2017). Population analysis suggested a history of population bottlenecks in VNBI, along with lineage-specific differences in the mating type loci (Desjardins et al., 2017).

2.2.2.3 *C. neoformans* microevolution

Cryptococcus isolates are able to generate phenotypic variation within micro-evolutionary timescales. Indeed, isolates belonging to VNI, VNII and VNB each showed microevolutionary changes following 124–290 days of hospital care including antifungal treatment (Chen et al., 2017). Various mechanisms of enhanced virulence were identified including growth at 39 °C, adaptation to stress, capsule production; and amplification of the ERG11 gene at both the native and unlinked locus likely providing stable resistance to fluconazole (Chen et al., 2017). Various genes exhibited copy number between serial isolates in addition to ERG11, including chitin deacetylases Cda2 and Cda3 that may alter the cell wall, as well as capsule-associated protein CAS33 (Chen et al., 2017). Thus, further efforts to study microevolution may reveal more targets for improved diagnosis, therapy, and prognosis.

2.2.3 The genetically distinct populations of C. gattii *(VGI → VGVI)*

2.2.3.1 *C. gattii* VGI, VGII, VGIII and VGIV

Since 2002 when the isolates of serotype B/C were formally classified as *C. gattii* (Kwon-Chung et al., 2002) and serotype A/D strains were classified as *C. neoformans* (Kwon-Chung & Varma, 2006), there has been substantial work on the population structure of *C. neoformans* and *C. gattii* using molecular typing methods such as PCR fingerprinting (Meyer et al., 2003), AFLP/RFLP analysis (Boekhout et al., 2001) and multi-locus sequencing (MLST) (Meyer et al., 2009), and more recently whole-genome sequencing (Cuomo, Rhodes, & Desjardins, 2018) which demonstrated that both species contain genetically diverse lineages that qualify them to be considered as two species complexes (Hagen et al., 2015; Meyer et al., 2003). Indeed, up until recently, *C. gattii* was thought to comprise only four distinct lineages (variety *gattii* (VG) I, II, III and IV) (Bovers, Hagen, Kuramae, & Boekhout, 2008). In 2019, a fifth lineage was confirmed from Zambia (VGV) and

a sixth from Mexico was shown to be a unique non-hybrid lineage (*C. decagattii*/VGVI) (Farrer et al., 2019).

Each of the lineages has such considerable genetic variation to warrant classification as separate species (*C. gattii*, *C. deuterogattii*, *C. bacillisporus*, *C. tetragattii*, and *C. decagattii* for VGI, VGII, VGIII, VGIV and VGVI, respectively) (Hagen et al., 2015). However, *C. gattii*'s subpopulations nomenclature are still being debated (Hagen et al., 2017; Kwon-Chung et al., 2017). While each lineage comprises a monophyletic clade that may be viewed as species under an evolutionary species concept (Wiley, 1978), the true extent of *Cryptococcus* diversity worldwide remains unclear (Hagen et al., 2017), as indicated by the discovery of VGV and VGVI (Farrer et al., 2019) and the discovery of further "intermediate" or extensive recombinants between them may erode the proposed species boundaries. We have therefore argued that the names "VN" and "VG" serve as a practical "zip-code" within *C. neoformans* and *C. gattii*, respectively, offering a convenient way to describe newly discovered lineages or recombinants without introducing unwanted nomenclatural instability and confusion (Farrer et al., 2019).

Genome sequencing and phylogenetic analysis for three of the four originally discovered lineages (VGI, VGII and VGIII) have revealed sub-structures within those lineages. For example, we found that the Zambian VGI isolates fell within a distinct subclade on VGI, which we named VGIb to distinguish it from other isolates from the Pacific North West and Australia that fell within VGIa. Included within VGIa is the WM276 isolate from woody debris of forest red gum (*Eucalyptus tereticornis*), which was one of the first to have its genome sequenced and assembled (D'Souza et al., 2011). VGII is divided into several sub-lineages including VGIIa, VGIIb and VGIIc. All three of these sub-lineages are represented in the 1999 outbreak of *C. gattii* that occurred on Vancouver Island in Canada, which subsequently expanded to the Pacific Northwest (Fraser et al., 2005; Hoang, Maguire, Doyle, Fyfe, & Roscoe, 2004). Specifically, VGIIa and VGIIc lineages are largely restricted to the Pacific northwest (Billmyre et al., 2014; Engelthaler et al., 2014), while VGIIb is found elsewhere including the isolate Ram5 found on a Darwin stringybark (*Eucalyptus tetrodonta*) tree in the Northern Territory of Australia in 1999 (Chen et al., 2000). VGIIx is another transcontinental clade (Farrer et al., 2015) and including an isolate from Piauí, Brazil in 1995 (Hagen et al., 2013; Meyer et al., 2003) as well as isolates from Athens, Greece in 1998 (Bovers et al., 2008). Additional VGII subclades

VGIIy and VGIIz were recently described, consisting of several environmental Zambian isolates (Farrer et al., 2019). VGIIz is currently only populated by isolates from Zambia, while VGIIy includes both Zambian and Australian isolates.

MLST and genome sequencing of *C. gattii* VGIII has similarly revealed two subgroups: VGIIIa and VGIIIb (Byrnes et al., 2011), which mostly corresponded with a separation of serotype B and serotype C isolates, respectively (Firacative et al., 2016). Both clades, which likely originated from endemic regions in Colombia, Mexico and the United States, include clinical, environmental and veterinary isolates (Firacative et al., 2016). To date, only four isolates of VGIV has been whole-genome sequenced (Farrer et al., 2019), with insufficient genetic divergence shown to establish further population structure.

The first *C. gattii* genomes sequenced were VGIa WM276 representing VGI and VGIIb R265, strains involved in the 1999 outbreak in the Pacific Northwest (D'Souza et al., 2011; Fraser et al., 2005). Later, 16 genomes representing four of the major molecular types of *C. gattii* (including five VGI, eight VGII, three VGIII and one VGIV isolates) were assembled and compared (Farrer et al., 2015). This multi-genome approach enabled the identification of 15 structural rearrangements between lineages, which were almost exclusive to the VGI-III-IV lineages. Using synteny to inform orthology prediction, we identified 87% of *C. gattii* genes were present as single copies across all four lineages. Other genes were variably inherited across lineages including genes involved in oxidative stress and mitochondrial import. We hypothesize that lineage-specific genes may result in yet-to-be-discovered phenotypic differences between the lineages.

2.2.3.2 *C. gattii* VGV and VGVI

In 2019, we reported the fifth lineage of *C. gattii* (VGV) (Farrer et al., 2019). VGV is currently represented by six isolates collected from a 430 km span of northern Zambia including the Mupata Hills (Luanshya, Copperbelt Province), Mutinondo wilderness area and Kapishya (Mpika, Northern Province), suggesting that the lineage has a broad regional distribution across the Miombo woodlands ecoregion that it was discovered from, and which covers much of Central and East Africa. The six isolates of VGV fell within two distinct subclades (A and B). Clade A comprises three VGV isolates that were recovered from soil and animal dung sampled in hyrax middens from which we also identified VGI and VGII isolates. Clade B isolates were recovered from two locations, the first from which two isolates were

recovered were from a site approximately 345 km away from clade A, and a third isolate was recovered from a second site approximately 430 km away from the other clade B isolates. Clade B isolates were recovered from both a tree hole and also hyrax middens, showing that the lineage can occupy both tree and dung-associated environments. The fact that clades A and B were found in different geographic locations might reflect a degree of spatial genetic structure within VGV. All the VGV isolates were located in regions of granite and acidic kopjes/hills that are found occurring patchily across this ecoregion.

Some VGV isolates were recovered from middens of the Southern tree hyrax (*Dendrohyrax arboreus*), which are small herbivores that are most closely related to elephants. Hyrax defecate in communal latrines called hyrax middens, usually located in crevices in rocky kopjes, over many generations (Scott, 1990). These locations are often sheltered in rocky caves and droppings are likely to accumulate for upwards of 50,000 years, in some cases forming a stable paleoenvironmental hotspot of urea-rich nitrogenous material and containing an unprecedented richness of paleoenvironmental proxies (Chase et al., 2012). *Cryptococcus* has a pronounced trophism for urea as a nutritive substrate including pigeon guano, which is known to support prolific growth of *C. neoformans* and to a lesser extent *C. gattii* (Nielsen et al., 2007). Our finding that hyrax middens are hotspots of *Cryptococcus* diversity (including VGI, VGII, and VGV from just our sampling in Zambia) suggests that their ecological stability in landscapes that are rich in nitrogen availability may lead to them being important arenas for the evolution of *Cryptococcus*, and will likely be fertile ground for further discovery of diversity within this genus.

VGV has to date only been acquired from environmental samples (Farrer et al., 2019). VGV is capable of causing mild lung infection with negligible neurotropism in mice. Indeed, environmental isolates of other lineages of *C. gattii* and *C. neoformans* have also shown to be less virulent in murine models than clinical isolates of the same molecular type (Litvintseva & Mitchell, 2009; Springer et al., 2014), suggesting that certain alleles or epigenetic factors may be responsible for differences in pathogenicity. While VGV has a large genetic distance from other lineages, it was indistinguishable from VGIV using the routine RFLP analysis (Meyer et al., 2003). Thus, it is possible that previous isolates belonging to VGV may have been misidentified as VGIV using non-WGS methods, which is the most prevalent lineage found in Africa (Chen et al., 2014). The most likely candidates for the search of clinical VGV are VGIV

serotype B isolates recovered from patients. Geographically, the most likely place to find the VGV clinical isolates appear to be in sub-Saharan Africa since the current panel of isolates were found in the Zambian environment within an ecoregion that includes Tanzania, Burundi, Democratic Republic of the Congo, Angola and Malawi. The search for VGV clinical isolates is needed in order to understand the true virulence potential of VGV and whether it can spillover into humans.

C. gattii VGVI (also described as *C. decagattii*) represents the sixth, and to date final lineage that has been identified within the *C. gattii* species complex (Farrer et al., 2019). Thus far, VGVI is the only lineage that exists as a single genotype since the three isolates previously designated as *C. decagattii* appear to have been isolated from the same patient from Mexico (Hagen et al., 2012). Correspondingly, we found the lowest nucleotide diversity (π) for any of the six lineages in VGVI (Farrer et al., 2019). Firacative et al. (2016) identified VGVI as a VGIII-like isolate, with subsequent genome sequencing confirming VGIII to be the closest relative of VGVI (Farrer et al., 2019). Accordingly, of the six lineages, VGIII and VGVI shared the most private alleles (92 kb total; 5.37 per kb), which account for an average of 12% of all SNPs (based on alignments to VGII) found in isolates from those lineages (Farrer et al., 2019).

Synteny was highly conserved between the newly described VGV and VGVI, although with some notable differences (Farrer et al., 2019). For example, VGV has a single 171 kb inversion on supercontig 7 compared with its nearest VGIII and VGIV relatives. VGVI also has some chromosomal translocations compared with its closest relative VGIII (Farrer et al., 2019). Most of the protein-coding genes in VGV and VGVI belonged to orthogroups with the other lineages. However, 74 lineage-specific genes were identified that were unique to VGV, including 2 sugar transporters, an alcohol dehydrogenase, and an aldehyde dehydrogenase. Further, 49 genes were uniquely absent in VGV including 8 uncharacterized transmembrane proteins. Similarly, VGVI encodes 80 lineage-specific genes, while uniquely having lost 127 genes. However, none of these lineage-specific or uniquely-lost genes are currently known to be involved in virulence or contributing to other phenotypes.

Nearly 1 in 10 nucleotides in the *C. gattii* genome has an alternative allele across the 6 lineages (1.55×106 sites; 9.01% of the *C. gattii* genome) (Farrer et al., 2019). Indeed, >180 kb of these unique/private alleles were identified for each lineage, including for VGV which had 220 kb private alleles (12.75 per kb). VGI is the most distinct in terms of both the highest

count of private alleles (378 kb/21.93 SNPs per kb) and its nucleotide diversity (π), which is reflected in the three distinct subclades of VGI isolates in the whole genome phylogeny.

Despite the genetic divergence and highly structured ancestry of *C. gattii* lineages, many have been shown to mate and exchange genetic material. Interestingly, hybridization in *Cryptococcus* can also be confined to the mitochondrial genome, and demonstrated by a VGII and VGIII in vitro cross (Voelz et al., 2013). *C. gattii* maintains the ability to form hybrids with *C. neoformans*, e.g., serotype BD hybrids such as VGI-VNI (Bovers et al., 2006) and VGII-VNIV (Aminnejad et al., 2012) hybrids. To date, *C. neoformans*/*C. gattii* hybrids or recombinants have not had their genomes assembled or been thoroughly examined for the loci of crossovers, and differences in gene content (i.e., lineage-specific genes), which may provide clues about the rarity of hybridization between them, and the functional consequences of hybridization.

2.3 Ecology of *Cryptococcus*

Cryptococcus neoformans and *C. gattii* has associations with a range of animals, both as a pathogen and an environmental saprobe on feces. For example, both *C. neoformans* and to a lesser extent *C. gattii* has been found growing on pigeon guano. Various studies have highlighted its niche for pigeon guano including 2.5–8.1% of samples in Isfahan, Iran (Isfahani, Shadzi, Pour, & Ilchi, 2001; Soltani, Bayat, Hashemi, Zia, & Pestechian, 2013), and 15% in various provinces in lower Egypt (The Nile Delta) (Refai, Taha, Selim, Elshabourii, & Yousseff, 1983). In Scotland, United Kingdom, two patients died from cryptococcosis in 2019 at the Queen Elizabeth University Hospital with pigeon droppings found in a non-public room within the hospital containing culturable *Cryptococcus* and blamed for the outbreak (BBC News, 2019). Other *Cryptococcus* species are also commonly found in association with pigeon guano including *C. uniguttulatus* and the rare human pathogen *Cryptococcus laurentii*, both of which were isolated from droppings and cloacal swabs of feral pigeons (*Columba livia*) in Malmö, Sweden (Mattsson et al., 1999).

There is a clear need for a systematic study determining if *Cryptococcus* can be recovered from pigeon guano worldwide, and if there are any differences in prevalence or clade between environments (e.g., urban vs forest). Importantly, there is currently no evidence that either *C. neoformans*

or *C. gattii* survives or causes disease in pigeons, possibly owing to their high body temperature of up to 44 °C during flight (Aulie, 1971), compared with only a few degrees above 37 °C during exercise for humans (Gleeson, 1998). Thus, it is thought that environmental *Cryptococcus* lands on and grows on the nitrogen rich guano rather than being transferred to it through the GI tract of the bird. Interestingly, *C. neoformans* is able to complete its life cycle (i.e., mating) on pigeon guano but *C. gattii* does not, indicating that pigeon guano could represent the realized ecological niche for *C. neoformans* (Nielsen et al., 2007). The authors of that study suggested that an ancestral *Cryptococcus* strain therefore must have gained the ability to sexually reproduce in pigeon guano and then swept the globe (Nielsen et al., 2007).

Both *C. neoformans* and *C. gattii* are found frequently in soil, leaves, decaying wood, and tree hollow. For example, *C. gattii* is often associated with various Eucalyptus species, which are sometimes suspected to spill-over into human infections, such as the three cases of cryptococcosis in Punjab, India in 1993, which were linked to flowers from two imported trees of *E. camaldulensis* that also had *C. gattii* (Chakrabarti et al., 1997). Sub lineages of VGII including VGIIb were involved in the Vancouver Island outbreak (Billmyre et al., 2014; Engelthaler et al., 2014), which also includes isolates such as Ram5 that was found on a Darwin stringybark (*Eucalyptus tetrodonta*) tree in the Northern Territory of Australia in 1999 (Chen et al., 2000). Indeed, most *Cryptococcus* species live in the soil and rarely cause infections in mammals. For example, common *Cryptococcus* species include *C. laurentii* and *C. albidus* that are generally considered saprobic. However, the incidence of infection predominantly of the skin, bloodstream, and central nervous system due to these species has increased in recent years (Khawcharoenporn, Apisarnthanarak, & Mundy, 2007). The most clinically significant risk factors for non-neoformans cryptococcal infections include impaired cell-mediated immunity, corticosteroid use, and invasive devises such as catheters (Smith, Sehring, Chambers, & Patel, 2017), indicating that like *C. neoformans* and *C. gattii*, they are largely opportunistic fungal pathogens, albeit much rarer.

In 2019, we also recovered the first isolates belonging to the lineage VGV, along with VGI and VGII isolates from Southern tree hyrax (*Dendrohyrax arboreus*) middens, midden soil and tree holes (Farrer et al., 2019). While *C. gattii* is yet to be shown to pass through the GI tract of mammals, such associations with small mammals such as Hyrax may suggest an evolutionary

mechanism to generate adaptations that confer pathogenicity—a hypothesis recently named the "endozoan, small-mammal reservoir hypothesis" (Taylor & Barker, 2019), which deserves to be explored further.

The evolution of virulence in *Cryptococcus*, as well as a range of other fungal pathogens including *Aspergillus* spp. and *Candida* spp., may have been driven by interactions and predation by free living amoeba species such as *Acanthamoeba castellanii*, in what has been called the "amoeboid predator-fungal animal virulence" hypothesis (Casadevall, Fu, Guimaraes, & Albuquerque, 2019). It has been known since the 1950s that *A. castellanii* feeds on *C. neoformans* (Castellani, 1955). The *Cryptococcus* polysaccharide capsule, melanin synthesis and phospholipase have each been shown to be important for *C. neoformans* to resist predation by *A. castellanii*, and *C. neoformans* responds to both *A. castellanii* and mammalian macrophages by enlarging, triggered by phospholipase-B expression (Chrisman, Albuquerque, Guimaraes, Nieves, & Casadevall, 2011). It will be insightful for future studies to focus on further aspects and commonalities between amoeba-fungal interactions and animal phagocytic cell-fungal interactions, along with the distributions of various natural predators of *Cryptococcus* such as amoeba, and any variation in phagocytic potential between species of predator or species/lineage of fungus.

2.3.1 *Geographical distribution of* Cryptococcus

C. neoformans and *C. gattii* are globally ubiquitous in both environmental and clinical settings. However, there is evidence for continental and even country-level population structure (i.e., lineage presence/absence or major differences in proportion). In the following section, we summarize some of this population structure, with a particular focus on the meta-study by Cogliati (2013), who analyzed publications in the PubMed database that included the word "*Cryptococcus*," identifying 68,811 *C. neoformans* and *C. gattii* isolates, reported by hundreds of global research studies.

C. gattii is reported less often than *C. neoformans* worldwide, with the notable exception of Oceania. It is unclear if there is a biological or evolutionary reason for this, or if it simply due to sampling biases. However, countries including Australia, New Zealand, Papua New Guinea, and Hawaii Islands had a greater proportion of *C. gattii* compared with *C. neoformans*. VGI represented the most frequently isolated molecular type isolated from clinical and environmental sources (39%), and together with VGII (22%) and VGIII (3%), make up the dominant species complex ($n = 1328/2228$; 64%) in this region (Cogliati, 2013). In contrast, *C. neoformans* ($n = 900/2228$) lineage VNI comprised just 27% of

environmental and clinical isolates, VNII comprised 7% of isolates, and VNIII and VNIV comprised just 2% (Cogliati, 2013).

Cryptococcus has been extensively reported across Asia ($n = 19{,}651$), with most (80%) from China, India, and Thailand (Cogliati, 2013). VNI comprised 81% of the Asian samples that had their lineage determined ($n = 1708/19{,}651$), with VNII, VNIII and VNIV making up 3.7% of isolates (Cogliati, 2013). *C. gattii* made up only a small percent of total environmental, clinical and veterinary isolates that were typed to the resolution of species complex ($n = 682/5874$, 11.6%). Of the 1708 isolates that had their lineage determined, VGI was the most commonly found (13.2%) followed by VGII (1.7%), VGIV (0.3%) and VGIII (0.1%) (Cogliati, 2013). *C. gattii* was primarily isolated from tree samples including *Syzygium cumini*, *Mimusops elengi*, *Azadirachta indica*, *Acacia nilotica*, *Cassia fistula*, *Manilkara hexandra*, *Polyalthia longifolia*, *Eucalyptus camaldulensis*, *Tamarindus indica*, *Cassia marginata*, and *Mangifera indica*. The 10 isolates from an animal source were recovered from koalas living in two different zoos in Japan, again revealing a likely ancient association with *Eucalyptus* (Cogliati, 2013).

Africa was the continent with the highest number of samples: 19,753 *C. neoformans* and *C. gattii* strains from 25 of the 58 African countries, albeit with a predominance of isolates from South Africa (79%) (Cogliati, 2013). Isolates primarily derive from birds' excreta, as well as imported/invasive trees (e.g., *Eucalyptus camaldulensis*) and endemic mopane trees, and baobab trees (Cogliati, 2013). Molecular typing techniques were applied to only 2% of isolates ($n = 505$). Of those typed, 68% were VNI, while VNII and VNIII represented 11% and 1% of the isolates, respectively (Cogliati, 2013). A further 13% of African isolates were VNB molecular type, which were originally identified in Botswana, but have since been found in South Africa, Rwanda, and Republic Democratic of Congo (Cogliati, 2013). The epidemiology of *C. gattii* appears to differ significantly in Africa compared with the rest of the world (Chen et al., 2014) with a predominance of clinical VGIV isolates. Environmental *C. gattii* isolates in Africa have been recovered from soil, *Eucalyptus camaldulensis*, and almond trees. Two veterinary isolates were also reported from two cases of cryptococcosis affecting South African cheetahs (*Acinonyx jubatus*). Given VGIV's close relationship to VGV and associated difficulty with identification using the *URA5* RFLP obtained by double digestion with *Sau*961 and *Hha*1 (Farrer et al., 2019), it is plausible that some VGIV infections are being caused by VGV.

Cryptococcus is found across Europe, with most reported from France, Spain, Italy, and United Kingdom (82% out of the total 8736 isolates) (Cogliati, 2013). Most environmental isolates of *C. gattii* were from

Eucalyptus camaldulensis, Douglas tree, carob tree, and stone pine, whereas *C. gattii* animal infections were reported in a ferret and in some goats (Cogliati, 2013). *C. gattii* were reported less commonly than *C. neoformans* in Europe (~4% of all *Cryptococcus* reported and typed). The most common lineage reported in Europe is VGI (Cogliati, 2013), followed by VGII (Chen et al., 2014). In Spain, VGI caused an outbreak in goats causing predominantly severe pulmonary disease (Baró, Torres-Rodríguez, De Mendoza, Morera, & Alía, 1998), while a small number of imported human cases of VGIIa have occurred following travel to Vancouver Island (Levy, Pitout, Long, & Gill, 2007).

Cryptococcus species were reported from Central and South America ($n = 10{,}548$), with 90% from Brazil (53%), Columbia (22%) and Argentina (15%). The majority of strains ($n = 8590$; 81%) originated from clinical sources, and the remaining from environmental and veterinary sources (Cogliati, 2013). *C. neoformans* were detected from a range of environmental sources (trees, birds' excreta, soil), as well as animal sources (insects, bulls and sheep) (Cogliati, 2013). *C. neoformans* was recognized in 6665 isolates (82%), compared with 18% for *C. gattii*, which was almost exclusively VNI (71% of isolates), while VNII, VNIII and VNIV (hybrid) comprised on 3.4% of isolates. There was also disparity between countries. For example, all major *Cryptococcus* lineages were recovered from countries such as Brazil and Columbia, which contrasts with Chile where all the four *C. neoformans* molecular types were reported, but no *C. gattii* isolates were found (Cogliati, 2013).

C. neoformans was more frequently isolated in North America than *C. gattii* ($n = 3148/4033$), despite the monitoring efforts that followed the Vancouver Island outbreak (Cogliati, 2013). Most *Cryptococcus* isolates that were reported from North America were from the United States (79%), but also Canada (15%), and Mexico (6%). Eighty percent were from clinical sources and the rest from environmental and animals (including pigeon excreta, fruit and vegetables, and ferrets). VNI is the prevalent molecular type (33%) in both the United States and Mexico, although VNII (1%), VNIII (5%), and VNIV (5%) are also present in lower percentages (Cogliati, 2013). *C. gattii* has been widely reported in North America, owing to Vancouver Island, Canada outbreak in 1999, which subsequently expanded to the Pacific Northwest (Fraser et al., 2005; Hoang et al., 2004). The outbreak was caused by VGII, specifically VGIIa, VGIIb and VGIIc. Surprisingly, while VGIIa and VGIIc lineages are largely restricted to the Pacific Northwest (Billmyre et al., 2014; Engelthaler et al., 2014), VGIIb

is also found elsewhere including in environmental isolates from the Northern Territory of Australia (Chen et al., 2000). Monitoring during the outbreak found that soil, trees, dogs, cats, horses, ferrets, and birds living in Vancouver Island have all been colonized by *C. gattii* (Bartlett et al., 2012). The most common type of *Cryptococcus* types in North America was VGIIa ($n = 473$, 39% of all isolates), a finding firmly established due to extensive efforts to track the source of the outbreak. Other minor clades from North America were VGIIb ($n = 57$; 5%), VGII other (0.2%), VGIII (4%) and VGIV (1%). The VGIV isolates were all reported from Mexico, and have not yet been identified in other North American countries (Meyer et al., 2003).

2.4 Virulence and pathogenicity factors of *Cryptococcus*

The ability to grow at mammalian temperatures and to form extracellular capsules are arguably the most important virulence factors described in *Cryptococcus* to date. Indeed, the ability to grow at 37 °C is a distinguishing feature of *Cryptococcus* compared with its closest saprobic relatives *T. wingfieldii* that doesn't grow above 24 °C and *C. amylolentus* that does not grow above 30 °C (Findley et al., 2009). Other virulence factors have also been identified including phospholipase-B, laccases which catalyze melanin synthesis, and urease have each been implicated in the ability of *Cryptococcus* to disseminate from the lung via the lymphatic system and blood to the CNS. Additional features of hypervirulent outbreak strains (such as the VGIIb clonal group) have an enhanced ability to rapidly proliferate within host macrophages where reactive oxygen species (ROS) stimulate tubular mitochondrial morphology in a subset of cells, which appears to be a protective mechanism from autophagic degradation. Some microevolutionary variants have been described that associate with these phenotypes (Farrer et al., 2016), but await further phenotypic validation. Indeed, a systematic comparison between all of the lineages for the presence or absence or genetic diversity of the genes implicated in each of these processes may give new clues to their evolution and function.

Cryptococcus are overwhelmingly isolated and studied in the yeast forms (Fig. 1A). In laboratory rich media they grow as regular yeasts and during mammalian infection it has not been observed to form invasive hyphae, like other medically-important fungi *Candida albicans* and *Aspergillus fumigatus*. However under stress conditions it possesses several morphotypes (Fig. 1). *C. neoformans* has the ability to enlarge its size during infection to form

Titan cells (growing from typically 5–7 μm cell body diameter to >10 μm, and in some cases up to 100 μm) (Okagaki et al., 2010; Zaragoza et al., 2010; Zaragoza & Nielsen, 2013) (Fig. 1F). Titan cells are induced in response to environmental stimuli consistent with the host lung, including nutrient starvation, 5% CO_2 and 37 °C, comprise up to 20% of the total in vivo cell population and have a number of hallmarks including altered capsule, cell wall, size, high mother cell ploidy, and aneuploid progeny (Okagaki et al., 2010; Zaragoza et al., 2010; Zaragoza & Nielsen, 2013). Importantly, Titan cells are too enlarged to be phagocytosed by macrophages and are more resistant to oxidative and nitrosative stress, and therefore contribute to fungal persistence during long time periods (Zaragoza, 2019). To date, Titan cells have been primarily studied in *C. neoformans*, with few reports from *C. gattii* (Dylag, Colon-Reyes, & Kozubowski, 2018). It is unknown if saprobic relatives of *C. neoformans* and *C. gattii* are able to generate Titan cells, although nine non-*neoformans* species did not form Titan cells in response to fetal bovine serum (Dylag et al., 2018). Indeed, other *Cryptococcus* morphotypes have been observed from the typical yeast such as hyphae and pseudohyphae when confronted with amoeba (Fig. 1C), hyphae following mating, and shmoos might each have various roles or be regulated in different ways during infection (Wang & Lin, 2015). In contrary to other yeasts such as *Candida* spp., morphotype regulation and signaling in *Cryptococcus* is relatively poorly studied.

Cryptococcus' polysaccharide capsule provides a physical barrier that interferes with normal macrophage phagocytosis and clearance by the immune system (Bose, Reese, Ory, Janbon, & Doering, 2003) (Fig. 1E). Differences in capsule size were reported between lineages and even between the different subclades VGIIa and VGIIb (Ngamskulrungroj, Price, Sorrell, Perfect, & Meyer, 2011). However, it is unclear if these phenotypic differences are due to the distribution or allelic-richness of virulence determinants and have significant functional consequences. Differences in capsule size have also been previously reported between lineages and even sub-lineages of *Cryptococcus gattii* VGII (Ngamskulrungroj et al., 2011). The size and morphology of the newly discovered VGV yeast cells were typical for *Cryptococcus* and indistinguishable from VGIV strains. However, two distinct patterns of capsule formation were found among the six VGV isolates grown in YEPD broth. The VGV clade A isolates recovered from soil produced a thinner capsule (≤1 μm) compared to those clade B isolates recovered from tree bark that produced thick (2–4 μm) capsules similar to the VGIV control strains (Farrer et al., 2019). In this particular

case, differences in capsule size did not however reflect differences in virulence in mice.

Cryptococcus encodes various enzymes that are recognized as virulence factors, since they can promote survival and replication in macrophages and/or enabling *Cryptococcus* to disseminate from the lung via the lymphatic system and blood to the CNS (Santangelo et al., 2004). *C. neoformans* and *C. gattii* encode five groups of phospholipases (A_1, A_2, B, C and D), with phospholipase-B (Plb) enzyme B1 (*PLB1*) and the phosphatidylinositol (PI)-preferring phospholipase-C (Plc) both implicated in pathogenicity in fungi (Djordjevic, 2010). Plb enzymes can be secreted/extracellular or remain intracellular, where they remove fatty acyl chains from glycerophospholipids, as well as physically disrupt host membranes, and/or by a number of other enzymatic activities affecting fungal cell signaling (acting as secondary messengers) and production of immunomodulatory effectors (Djordjevic, 2010). Particularly, this disruption of host membranes may be the single responsible for the observed effects that *plb*Δ mutants are unable to disseminate effectively to the central nervous system (Santangelo et al., 2004).

Cryptococcus encodes two laccases (*LAC1* and *LAC2*) which catalyze melanin synthesis (Qiu et al., 2012) and are tightly associated with the cell wall where they oxidize both polyphenolic compounds and iron (Zhu, Gibbons, Garcia-Rivera, Casadevall, & Williamson, 2001). Laccase activity in *C. neoformans* is induced by a variety of environmental stimuli including metals (Ca^{2+}, Fe, Cu), low concentrations of glucose and nitrogen, and elevated temperature (Zhu & Williamson, 2004). Laccases are responsible for a diverse array of functions both in the environment and the host. Within the environment, glucose deprivation leads to laccase expression that facilitates the breakdown of lignin (a foraging response in the hollows of trees) via its enzymatic activity (Zhu & Williamson, 2004). Importantly, laccases are also able to convert host-derived substrates into melanin, which protect the fungus from host attack. For example, laccases convert catecholamine neurotransmitters such as dopamine into melanin by oxidizing it to the highly reactive o-quinone (DAQ) (Zhu & Williamson, 2004). The resulting melanin provides cellular protection both environmentally and in the host. For example, melanin offers protection from ultraviolet light and temperature extremes from sunlight warming (Wang & Casadevall, 1994). Melanin also provides protection from ingestion and killing by alveolar macrophages, as well as killing by antimicrobial peptides (Casadevall, Rosas, & Nosanchuk, 2000). *LAC1* and *LAC2* are both induced upon

exposure to murine macrophages (Farrer et al., 2018), but *LAC1* having dominant enzyme activity under glucose starvation (Zhu & Williamson, 2004), while *C. gattii* VGII switches expression from *LAC1* in vitro to LAC2 (peaking at 3 h post coincubation with murine bone marrow derived macrophages) (Farrer et al., 2018).

Cryptococcus encodes an extracellular-bound urease (by the gene *URE1*), which are Ni(2+)-dependent metalloenzymes that hydrolyze urea to produce ammonia and CO_2. Urease has a role in the survival and/or multiplication of yeasts within the macrophages (Feder et al., 2015) and was shown to be necessary to survive in pH above 6, and therefore has a fitness defect in the host extracellular milieu (Fu et al., 2018). This may explain why urease had been previously implicated in yeast crossing of epithelial and endothelial barriers (Feder et al., 2015). Other extracellular enzymes encoded by *Cryptococcus* that are thought to play roles in virulence or host-interactions include esterases, esterase lipase (C8), leucine arylamidases, acid phosphatases, alpha-glucosidase and beta-glucosidase (Almeida, Wolf, & Casadevall, 2015; Vidotto et al., 2006).

Subclades from the Vancouver Island outbreak (primarily *C. gattii* VGIIa and VGIIb) showed increased virulence. Several explanations were proposed for the observed hypervirulence: an enhanced ability to rapidly proliferate within host macrophages (Voelz et al., 2014), but no definitive explanation has been established. *C. gattii* is further able to protect itself from ROS and other host-imposed stresses such as iron deprivation (Vartivarian et al., 1993) and increased CO_2 concentrations (Granger, Perfect, & Durack, 1985) by encapsulating itself.

2.4.1 Sources of genome plasticity

Pathogenic fungi often manifest highly plastic genome architecture in the form of variable numbers of individual chromosomes (chromosomal copy number variation; CCNV; aneuploidy) (Farrer & Fisher, 2017; Farrer et al., 2013). Variation in chromosome copy number has previously been shown to influence the virulence of *Cryptococcus* (Hu et al., 2011) and can further provide resistance to azole drugs by increasing the copy number of the azole drug target (ERG11) or transporter (AFR1) commonly amplified in drug-resistant *Cryptococcus* (Kwon-Chung & Chang, 2012) and across the time-scale of a single infection (Rhodes, Beale, et al., 2017). Because CCNV is increased during mammalian infection or when exposed to antifungal treatment is seems to be a beneficial adaptation strategy to increase the virulence of the pathogen (Kwon-Chung & Chang, 2012).

C. neoformans and *C. gattii* both exhibit CCNV in clinical isolates (Hu et al., 2011; Lengeler, Cox, & Heitman, 2001), as well as being a characteristic for Titan cell (Okagaki et al., 2010; Zaragoza et al., 2010; Zaragoza & Nielsen, 2013). For example, both veterinary and clinical isolates belonging to VGII and VGIII showed evidence for CCNV (Farrer et al., 2015) including an additional (disomic) copy of scaffold 13 in VGII veterinary isolate B8828 and a disomy of scaffold II in VGIII clinical isolate CA1280.

Intrachromosomal CNVs have also been reported in *Cryptococcus*, such as a 60 kb intrachromosomal duplication found in middle of scaffold 1 of VGII isolate LA55 (from CSF male human, Piauí, Brazil, 1995 (Meyer et al., 2003)) in contrast to the otherwise genetically similar isolate CBS10090 (from the skin of a HIV-negative human in Athens, Greece, 1998 (Bovers et al., 2008)), suggesting it arose recently during infection (Farrer et al., 2015). The importance or effect of that 60 kb CNV is unclear, but covers 24 protein-coding genes that are not known to influence drug resistance in *Cryptococcus*.

Cryptococcus' chromosomes have been shown to fuse, split, and undergo inversions and translocations, which can have a dramatic effect on their phenotype and virulence. One method to study this in silico is to identify orthologs, and then to map their synteny. Chromosomal structure is mostly conserved among the six lineages of *C. gattii* (Farrer et al., 2019, 2015), and very highly conserved within VGII. Almost all syntenic variation was identified among the non-VGII isolates. Comparisons between VGI, VGII and VGIII and VGIV revealed 15 rearrangements included 10 translocations (7 inter-chromosomal and 3 intrachromosomal) and 5 scaffold fusions (Farrer et al., 2015), most of which (13 of the 15) associated with clusters of predicted *Cryptococcus*-specific TCN transposons found at centromeres (Janbon et al., 2014), suggesting these are primarily whole chromosome arm rearrangements. These changes may impact the ability for inter-lineage genetic exchange, as some crossover events will generate missing chromosomal regions or other aneuploidies and nonviable progeny.

Epigenetics is predicted to play an important role for heterogeneity in virulence, morphotypes and ecology in *Cryptococcus*, but research in this topic is still in its early years. For example, *Cryptococcus* encodes several class I/II histone deacetylases (HDACs) responsible for chromatin remodeling, and deletion of HDACs indicated they control a variety of cellular processes associated with virulence including thermotolerance, capsule formation, melanin synthesis, protease activity, mating, and cell wall integrity

(Brandão et al., 2018). Therefore HDAC deletion strains were less virulent during macrophage infection and in *Galleria mellonella* (Brandão et al., 2018). Gene expression in *Cryptococcus* is also regulated by miRNA. For example, RNAi-mediated gene silencing is active during mating in *C. neoformans* (sex-induced silencing) to limit or prevent gene disruption caused by various retrotransposons (Wang et al., 2010). It is noteworthy that *C. gattii* VGII is missing PAZ, Piwi, and DUF1785 domains, all of which are components of the RNAi machinery. This loss of RNAi has been hypothesized to contribute to increased genome plasticity in this lineage that may have contributed to specific hypervirulent traits in VGII (D'Souza et al., 2011, p. 2; Farrer et al., 2015; Wang et al., 2010).

3. Cryptococcosis

3.1 Exposure, latency and the various hosts of *Cryptococcus*

Cryptococcal spores and yeast can become airborne from environmental reservoirs such as tree hollows and leaf litter (May, Stone, Wiesner, Bihanic, & Nielsen, 2016; Springer et al., 2014). Airborne particles with an estimated size of >7 to 4.3 μm were detected in British Columbia (Kidd et al., 2007). Mammals often inhale these aerosolized yeasts or spores, which is their most common exposure route of *C. neoformans*. In mouse models, respiratory inoculation of spores and yeast cells have the same virulence characteristics indicating they both have the capacity to travel and disseminate through the respiratory tract to establish infection in mammalian hosts (Giles, Dagenais, Botts, Keller, & Hull, 2009). In British Columbia, 33% of air samples as well as freshwater (Kidd et al., 2007) had detectable levels of *Cryptococcus*. Infectious particles of *Cryptococcus* can persist in a variety of environments for several months. For example, *C. neoformans* survived up to 9 months in pigeon droppings in dry and hot environmental conditions of Oklahoma city, and can survive up to 1 year if stored at 4 °C (Ruiz, Neilson, & Bulmer, 1982). Water courses are another environmental reservoir of *Cryptococcus* since *C. gattii* yeasts remain viable for several months in water at room temperature of 21–23 °C, and up to 3 months if water was kept at 4 °C (Kidd et al., 2007). Traumatic inoculation from the soil contamination has been observed, and may be more frequent in animals that regularly sustain non-lethal injuries such as by fighting (Malik et al., 2011).

Presence of yeast particles suspended in the air leads to frequent exposure in humans. For example, up to 80% of 5-year-olds in the Bronx, a borough of New York City, United States manifest serological reactivity to *C. neoformans*, consistent with prior exposure (Davis et al., 2007; Goldman et al., 2001). Similarly, feral and companion animals are frequently carriers of pathogenic species of *Cryptococcus* in their upper respiratory tract, as well as their ears. Additionally, Animals are possibly more directly exposed than humans from the environmental reservoirs due to their foraging, feeding and fighting behaviors, e.g., up to 90% of feral cats in Italy were positive for *C. neoformans* (Danesi et al., 2014).

The infectious particles of *Cryptococcus* can travel through the respiratory tract of animals. Animals, particularly cats, manifest rhinitis and ear infections showing a persistence in all the upper respiratory tract and which may progress to life-threatening infections (Malik et al., 2011). Infections which persist in the upper respiratory tract is similarly common in infected laboratory animals. Intranasal instillation of guinea pigs (Lima & Vital, 1994) and mice (Coelho, Camacho, Salas, Alanio, & Casadevall, 2019) observed yeast proliferation in nares, which in the case of laboratory mice was accompanied by a quick invasion of the brain (under 3 h).

Mycobiome studies have not detected pathogenic species of *Cryptococcus* in the upper respiratory tract of humans (with one notable exception discussed below) (Chalermwatanachai, Zhang, Holtappels, & Bachert, 2015; Charlson et al., 2012; Cleland et al., 2014; Jain, Das, Gupta, & Malik, 2013; Jung, Croll, Cho, Kim, & Lee, 2015; Mac Aogáin et al., 2018; Ponikau et al., 1999; Ragab, Clement, Vincken, Nolard, & Simones, 2006). Culture methods and the now obsolete pyrosequencing methods found no presence of *Cryptococcus* in the respiratory tract of humans (Boase et al., 2013; Cleland et al., 2014; Ponikau et al., 1999; Ragab et al., 2006). In contrast, this genus is frequently identified in human mycobiome studies. One caveat of these studies is that they rarely identify below genus level and therefore there is little information in the species found in healthy human body, and preventing the distinction of pathogenic vs non-pathogenic species. The nasal vestibule mycobiome of a population from Seoul, Korea showed a high frequency of *Cryptococcus* spp., second only to *Malassezia* (Jung et al., 2015). *Cryptococcus* spp. were identified from skin, but not nares, of healthy children from Washington, DC, United States (Jo et al., 2016) and in breast milk of healthy women From China, South Africa, Finland and Spain (Boix-Amorós et al., 2019). A singular study found *C. neoformans* to be the most abundant fungal

species of the human nostril's mycobiome, present in the middle meatus in 60% of healthy controls and 90% of chronic rhinosinusitis patients from a population of chronic rhinosinusitis patients and controls from St. Louis Missouri (Aurora et al., 2013). The reasons for the high prevalence in this study when compared to the study from Korea are still unexplained. Clinically the presence (or absence) of *Cryptococcus* in human upper respiratory tract is mostly silent, as there are no known associations of rhinitis with *Cryptococcus* in humans, and laryngeal cases of cryptococcosis in humans are rare, with less than 30 case reports so far (Quintero et al., 2019), and the vast majority of the cases likely caused by inhaled corticosteroid use.

The difference in clinical manifestation of *Cryptococcus* in the upper respiratory tract of humans vs animals may have several explanations. Behavioral differences due to foraging likely lead to a more frequent exposure of animals vs humans. Most animals have larger and more complex noses that may better filter and retain infectious particles in the nares (Malik et al., 2011), leading to an increase of upper respiratory tract infections, while in humans the smaller nose may allow more infectious particles to reach the lung. It is not known if immunological differences may have a partial contribution for the different tissue preferences and clinical manifestations. For example, rabbits have a higher body temperature than humans which could explain relative resistance to infection (Perfect, Lang, & Durack, 1980). Notably, an increase in disease in animals is an important early alarm of a human outbreak. During the Vancouver outbreak an increase in frequency of veterinary *Cryptococcus* cases was noted and preceded human infections (Malik et al., 2011).

In summary, mycobiome studies show that species of *Cryptococcus* exist in small amounts in human mouth and skin (Aurora et al., 2013; Chalermwatanachai et al., 2015; Charlson et al., 2012; Cleland et al., 2014; Jain et al., 2013; Jung et al., 2015; Mac Aogáin et al., 2018; Ponikau et al., 1999; Ragab et al., 2006). Frequent exposure to *Cryptococcus* allows yeasts lodging in humans while kept in check due to effective control by the immune system. It remains unclear which species exist in association with humans and how the environmental reservoirs and exposure, followed by interactions with host immune and the human-associated microbial communities affect development of fungal disease.

Primary infection of humans by *Cryptococcus* is believed to be asymptomatic or to occur unnoticed as one of the many illnesses of childhood (Goldman et al., 2001). The current prevailing framework is that *Cryptococcus* infection is not completely cleared and instead it persists in

a latent asymptomatic state. This is supported by a range of studies. Epidemiological data in the early 90s found that *Cryptococcus* isolated from patients belong to serotypes most common at the patient's country of birth (Dromer, Ronin, & Dupont, 1992; Garcia-Hermoso, Janbon, & Dromer, 1999). Further, clinical data shows latency of pathogenic species in several internal organs of humans. Latency occurs commonly within the lung, the site of primary infection (Henao-Martínez & Beckham, 2015; Kanj et al., 1996; Lindell, Ballinger, McDonald, Toews, & Huffnagle, 2006), while latency in other tissues has been documented. Lung transplant recipients have developed cryptococcosis stemming from infections of the donors lung (Henao-Martínez & Beckham, 2015; Kanj et al., 1996). Similarly, some cases of cryptococcal disease were attributed to transmission of yeasts from the kidney and liver transplants to the organ recipient (Baddley et al., 2011; Mac Ewen, Ryan, & Winearls, 2013). Latency of cryptococcal infection also occurs in other mammalian (non-human) hosts. Companion animals show reactivation of infection (Kluger et al., 2006) and necropsy studies have shown subclinical cryptococcal granulomas in several animal species (Malik et al., 2011). In the rat model it is possible to establish latent infections that can be re-activated by subsequent immune suppression (Goldman, Lee, & Casadevall, 1994; Goldman, Lee, Mednick, Montella, & Casadevall, 2000). Therefore there is convincing proof of latency of dormant *Cryptococcus* throughout the body of infected mammals, particularly the respiratory tract. This demonstrates that prophylactic interventions are valuable in certain patients, for example, before a transplant or prolonged immunotherapy.

The observation of latent yeast cells in several organs in humans as well as in experimental models reveals a poorly understood facet of virulence: that yeasts possess a remarkable capacity to invade and disseminate throughout the entirety of the host body. In laboratory mice, *C. neoformans* spores and yeasts disseminate in less than 24 h to the mouse lymph nodes, albeit yeast forms slightly slower than spore forms (Walsh et al., 2019). Both *C. neoformans* and *C. gattii* yeast particles disseminate in a matter of hours to the mouse brain after deposition into mouse nares (Coelho et al., 2019). Once in the bloodstream, free yeasts become trapped within small capillaries (Shi et al., 2010). If these capillaries are within the host brain, the yeast will invade the underlying brain tissue (Shi et al., 2010). In contrast, if free yeasts arrest in the liver capillaries, then Kupffer cells (the liver resident macrophages) efficiently clear fungal cells from the bloodstream and arrest

fungal growth (Sun et al., 2019). These studies are the first to shed light on mechanisms of tissue invasion by *Cryptococcus*.

In the event of host immunosuppression, latent yeasts begin to proliferate until they perturb host tissue function and disease becomes noticeable (regardless of the latency site). In transplant patients, 0.25–5% develop cryptococcosis (Baddley et al., 2019; Kanj et al., 1996, p. 9), while in AIDS patients 6% of the patients are positive for cryptococcal antigen, with a staggering 15% of AIDS-related deaths being attributable to cryptococcosis (Rajasingham et al., 2017). As discussed above, up to 30% of HIV-associated cryptococcal meningitis may be attributed to *C. gattii* (Hurtado et al., 2019; Springer et al., 2014) and the remaining to *C. neoformans* species.

3.2 Antifungal treatment

Treatment for *Cryptococcus* infection generally consists of 2 weeks of induction therapy followed by 2 months of consolidation and several months of maintenance therapy. Induction therapy consists of a combination of amphotericin B (AmpB) with 5-fluorocytosine (5-FC), a pyrimidine nucleoside analogue of nucleic acids (Perfect et al., 2010; Spec, Mejia-Chew, Powderly, & Cornely, 2018), which is followed by several months of fluconazole as consolidation/maintenance therapy. Antifungal treatment lasts a minimum of 6 months, while in some patients eradication of fungus is not achieved and patients need lifelong antifungal therapy. Antifungal drug therapy is generally the same for all patients. Auxiliary measures, such as controlling elevated intracranial pressure, are also available and benefit the patients (Rolfes et al., 2014). Despite adequate medical care, fatalities still amount to 10–25% of the cases (Bratton et al., 2012; Hoang et al., 2004; Jarvis & Harrison, 2007; Rajasingham et al., 2017).

Management of cryptococcosis is difficult. AmpB therapy is more effective than fluconazole and other azoles, however treatment with amphotericin B is usually accompanied by severe side effects, in particular kidney failure (Longo et al., 2011). Its high toxicity and financial cost prevents prolonged courses of treatment and its use in resource-limited settings. In resource-poor settings, the toxicity of AmpB is compounded further as there are limited resources to prevent and handle its toxicity. If AmpB or 5-FC are not available (in resource-poor settings) then fluconazole at a high dose can be administered during the induction period. However monotherapy has suboptimal outcomes and allows the emergence of antifungal resistance (Hope et al., 2019). To mitigate the high costs of AmpB, the ACTA clinical

trial (ACTA-Advancing cryptococcal meningitis treatment for Africa) studied the effectiveness of shorter courses of AmpB and ideal timing of substitution for fluconazole, maintaining efficacy. They found that 1 week of AmpB combined with 5-FC was associated with the lowest 1-year mortality (Kanyama et al., 2019; Molloy et al., 2018).

AmpB binds to ergosterol causing pores and cellular leaking through the fungal cell membrane. Azoles inhibit lanosterol 14 α-demethylase, a critical intermediate in ergosterol biosynthesis, but are only fungistatic. *Cryptococcus* spp. are naturally resistant to echinocandins (which target β-glucan synthesis) and therefore these are not options for treating cryptococcal disease (Feldmesser, Kress, Mednick, & Casadevall, 2000; Maligie & Selitrennikoff, 2005). Each of the antifungal drugs used to treat *Cryptococcus* have existed for more than 30 years (Perfect et al., 2010; Sitapati et al., 2010; Spec et al., 2018), highlighting the difficulty in engineering drugs that target these remarkable fungi. In developing auxiliary therapies, there is a particular need for strategies to reduce treatment toxicity and duration and which increase fungicidal activity of azoles.

Individuals who develop cryptococcosis can be grouped in three specific risk groups: those with advanced HIV infection, those with organ transplants and associated immunosuppressive therapy (Baddley et al., 2019), and lastly non-HIV and non-transplant patients without an obvious immune disorder (Perfect et al., 2010). Another significant risk factor is liver disease (Singh, Husain, De Vera, Gayowski, & Cacciarelli, 2004). Importantly, cryptococcal meningitis is commonly overlooked in differential diagnosis in non-HIV patients, which leads to an increased time to diagnosis which negatively affect the prognosis and outcomes (Aye, Henderson, Yu, & Norton, 2016; Bratton et al., 2012; Katchanov, von Kleist, Arastéh, & Stocker, 2014; Perfect, 2013; Yoon, Felsen, Wang, & Pirofski, 2019). To prevent this delay in diagnosis it is critical to raise awareness in clinicians regarding invasive fungal infections in non-HIV population.

The differences between the three general patient risk groups detailed above are in how to address the underlying cause of immunosuppression. HIV + patients will be administered anti-retroviral therapy (ART). In organ transplants recipients and other patients undergoing immunosuppressive treatment, immunosuppressive treatment needs to be carefully monitored and may be reduced in a step-wise manner, as abrupt withdrawal can negatively skew the immune response to immune reconstitution syndrome (IRIS) or organ rejection (Perfect et al., 2010). Patients who are HIV- and without known immunosuppression regimens should be investigated

for underlying immune defects (such as decreased CD4 T-cells, IFNγ levels, etc.), as there are now immunotherapies available to address the underlying immunosuppression and greatly aid resolution of disease (see below).

One paradoxical manifestation of the disease is IRIS which is a clinical worsening due to deregulated host immune response to *Cryptococcus*. Although this syndrome is still poorly understood, its appearance in HIV patients has been associated with high cryptococcal burden accompanied with decreased levels of inflammatory cytokines in the CSF prior to ART initiation (Boulware et al., 2010), poor recovery of CD4 T-cell after ART (Chang et al., 2013), and high plasma IL-5 and IL-7 levels (Akilimali et al., 2017), as well as low IgM levels (Yoon, Nakouzi, et al., 2019). Management and diagnosis of IRIS are difficult clinically. Usual treatments are corticosteroids for a limited time, but the efficacy of these treatments has not been formally evaluated. In one case report, monoclonal antibodies blocking inflammatory molecules successfully dampened the devastating inflammation underlying IRIS (Sitapati et al., 2010).

3.2.1 Novel antifungal drugs, immunotherapy, antibodies and vaccines

Drugs targeting neurotransmitters. Some off-patent drugs have been tested as possible adjuvants for cryptococcal therapy. The antidepressant sertraline reduced fungal burden in mice to levels similar to fluconazole (but not as efficiently as AmpB) (Zhai, Wu, Wang, Sachs, & Lin, 2012). Results from clinical trials phase I/II were encouraging. Phase III trials in Mexico showed that sertraline as adjunctive to combination therapy of AmpB and fluconazole had no advantage in patients administered sertraline vs regular therapy (Villanueva-Lozano et al., 2018).

Cytochrome inhibitors CYP51 inhibitors VT-1129 and VT-1158, developed by Mycovia Pharmaceuticals (previously Viamet), inhibit ergosterol synthesis and are effective antifungal in animal models (Wiederhold et al., 2018). The arylamidine T-2307 (Toyama Chemical Company) which disrupts mitochondrial function in *C. neoformans* (Mitsuyama et al., 2008) and *C. gattii* (Nishikawa et al., 2017) has shown enough promise to warrant clinical trials.

Calcineurin pathway inhibitors: Inhibitors of the calcineurin pathway are potent immunosuppressive drugs in mammals and are used in transplant recipients to promote graft tolerance. It was noted that this therapeutic regimen in transplant patients affects susceptibility and mortality to cryptococcal disease (Kontoyiannis et al., 2008; Singh et al., 2007). Indeed the

calcineurin pathway is critically required for most virulence traits of *C. neoformans* (Steinbach, Reedy, Cramer, Perfect, & Heitman, 2007) and *C. gattii* (Chen, Lehman, Lewit, Averette, & Heitman, 2013). Very recently, the Heitman laboratory developed fungal-specific calcineurin inhibitors via rational drug design. By determining the crystal structure of fungal calcineurins (of *C. neoformans*, *A. fumigatus* and *C. albicans*), this group engineered small chemicals to bind fungal calcineurin with more affinity than mammalian calcineurin. These new chemicals show increased specificity over host immunosuppressive effects (in a model of NP-OVA antigen immunization) (Juvvadi et al., 2019), showing considerable potential as a new class of antifungal drugs.

3.2.1.1 Immunotherapy

Interferon-γ (IFNγ). In human patients a strong IFNγ cytokine response indicates a good prognosis (Jarvis et al., 2012). Clinical trials administering recombinant interferon-gamma (rIFNγ) to patients have been performed. The first study found "a trend toward better clinical outcome and earlier CNS sterilization but no differences in survival" (Pappas et al., 2004). Later a randomized controlled trial confirmed that administration of IFNγ improved fungal clearance from the CNS, but did not affect patient mortality (Jarvis et al., 2012). Therefore IFNγ therapy is not widely used in clinics (even when available), except in patients with verifiable lower levels of IFNγ and which benefit from administration of recombinant cytokine (Netea et al., 2004). The lack of beneficial results with IFNγ therapy should not be discouraging as it simply illustrates the need for better understanding of timing, dosage and tissue requirements for IFNγ. In mouse models, vaccines which rely in location dependent administration of IFNγ are very efficacious. Wormley et al. engineered a strain of *C. neoformans* to express mouse IFNγ, dubbed H99γ. When animals are infected with this strain they are protected from subsequent challenge with virulent strains and achieve sterilizing immunity (Wormley, Perfect, Steele, & Cox, 2007). This immunity is cross-reactive for multiple serotypes of *Cryptococcus* and dependent on macrophage iNOS and Stat1 (Leopold Wager, Hole, Wozniak, Olszewski, & Wormley, 2014; van Dyke et al., 2017). Therefore production of IFNγ at the site of infection is highly beneficial to the mammalian host. Overall these results are encouraging for a therapeutic role of IFNγ while also highlighting a need for a deeper understanding on dosing of IFNγ to elicit an optimal immune response.

Other immunomodulators. There are still no definitive recommendations for immunotherapy in cryptococcal infection. In HIV negative, non-transplant patients, a study of the patients may uncover idiopathic immune defects that can be targeted on a case by case basis (Netea et al., 2004; Yilmaz-Demirdag, Wilson, Lowery-Nordberg, Bocchini, & Bahna, 2008), for example IFNγ or IL-2. In patients who develop IRIS corticosteroids can be used to dampen inflammation.

A different type of immunomodulatory therapy is administration of mimics of pathogen associated molecular patterns (PAMPs), particularly TLR agonists. These microbial-molecule analogs are effective by eliciting a protective IFNγ response. Administration of TLR agonists, such as unmethylated CpG nucleotides (Kinjo et al., 2007; Miyagi et al., 2005) or polyinosinic:polycytidylic acid (poly-IC) a mimic of microbial RNA (Sionov et al., 2015), delayed mouse death upon *Cryptococcus* infection.

3.2.2 *Drug-resistance mechanisms of* Cryptococcus

C. neoformans may manifest resistance to all major classes of drugs (reviewed in Perfect & Cox, 1999). The development of resistance to AmpB during the course of clinical treatment has been observed, but it is relatively rare. 5-FC resistance is so common that it precludes its use as monotherapy, with the quick appearance of mutations in nucleotide metabolism that prevent 5-FC toxicity (Billmyre, Applen Clancey, Li, Doering, & Heitman, 2020). *Cryptococcus* frequently derives subpopulations intrinsically resistant to azoles used during treatment (Sionov, Chang, & Kwon-Chung, 2013). Heteroresistance may achieved through dormancy-like state, since in murine infection we can detect viable non-culturable yeast forms (Hommel et al., 2019), as well as chromosomal instability and aneuploidy (Hope et al., 2019; Stone et al., 2019).

There have not been any new classes of antifungals since the discovery of amphotericin B in the 1970s. While several drugs are currently being developed (Mourad & Perfect, 2018), a concerted effort of funders, researchers and pharmaceutical industry is required to bridge this gap in development of antifungal drugs.

3.3 Genetic risk factors underlying susceptibility to cryptococcosis

The mammalian immune defense against cryptococcosis is critically dependent on CD4-T cells and the mononuclear phagocyte system, macrophages

and monocytes, as well as B-cells (Coelho, Bocca, & Casadevall, 2014; Garelnabi & May, 2018; Mukaremera & Nielsen, 2017). The mouse immune system has proved a good model for human cryptococcosis, as the mouse model mostly recapitulates human susceptibility despite some limitations, such as the lack of widely used HIV- infection model. Below we detail the genetic factors increasing susceptibility to cryptococcosis in humans (Table 1). We expect that in the near future the advances and lower costs in genome technology will empower larger scale studies to provide an even finer detail on the genetic variants and risk factors to fungal diseases.

Table 1 Gene deficiencies associated with susceptibility to cryptococcosis in humans.

Gene	References
MBL2 LOF	Eisen, Dean, O'Sullivan, Heatley, and Minchinton (2008) and Ou et al. (2011)
IFNGR1 LOF and anti-IFNγ autoantibodies	Bustamante, Boisson-Dupuis, Abel, and Casanova (2014) and Chetchotisakd, Anunnatsiri, Nithichanon, and Lertmemongkolchai (2017)
IL12RB1 LOF and other Mendelian susceptibility to mycobacterial disease	Jirapongsananuruk et al. (2012)
FcγReceptor2/3 variants	Hu et al. (2012), Meletiadis et al. (2007), and Rohatgi et al. (2013, 2017)
Anti-GM-CSF autoantibodies	Applen Clancey et al. (2019), Crum-Cianflone, Lam, Ross-Walker, Rosen, and Holland (2017), Kuo et al. (2017), Rosen et al. (2013), and Saijo et al. (2014)
GATA2 LOF	Vinh et al. (2010)
STAT3 LOF (hyper-IgE syndrome)	Holland et al. (2007)
CD40L LOF (X-linked hyper-IgM syndrome)	Winkelstein et al. (2003)
STAT1 GOF	Toubiana et al. (2016)
NEMO/IKKG LOF (X-linked Mendelian susceptibility to mycobacterial disease)	Bustamante et al. (2014) and Panackal et al. (2017)

GOF, gain-of-function variants; LOF, loss-of-function variants.

3.3.1 Defects in immune cell function affecting multiple immune populations

Some genetic defects causing immune defects lead to broad susceptibility to fungal infections, including *Cryptococcus*. For example, human *STAT1* gain-of-function (GOF) variants predisposes mycobacterial infections as well as a breadth of fungal infections, including *Cryptococcus* (Toubiana et al., 2016). *STAT3* loss-of-function (LOF) (or hyper-IgE syndrome) also increases susceptibility to a wide range of fungal infections (Holland et al., 2007). Part of the JAK-STAT transcription pathway, both *STAT1* and *STAT3* are critical for immune cell signaling, particularly macrophages and B-cells, and any perturbation of their function creates a severe impairment of immune function. The X-linked hyper-IgM syndrome caused by deficiency in CD40L, a widely expressed T-cell co-stimulatory molecule, results in hyper-production of IgM, accompanied by defective T-cell function which results in early (prior to 1 year in age) severe infections by most bacterial and fungal pathogens (Winkelstein et al., 2003). Loss-of-function in the *GATA2* transcription factor is a common cause of MonoMac syndrome. Because *GATA2* is required for immune cell development and self-renewal loss of function in this gene causes a decrease in circulating monocytes, B-cells, and NK cells leaving the patient susceptible to a wide range of infections, in particular mycobacteria and *Cryptococcus*.

Nevertheless, there are instances where the susceptibility to fungal infections is more specific, i.e., when susceptibility to fungal diseases is not accompanied by susceptibility to other cryptococcal disease. Dectin-1 is the receptor which recognizes β-glucan, a fungal cell wall component. Dectin-1 LOF variants lead to chronic mucocutaneous candidiasis in humans and mouse models, but are not associated with cryptococcal infection (Brown, 2006; Nakamura et al., 2007; Walsh, Wuthrich, Wang, Klein, & Hull, 2017). A recent study found that in mice Card9 is essentially required for immunization with a vaccine strain LW10, suggesting that variants in Card9 may be relevant for long-term protection against cryptococcosis (Campuzano et al., 2020). Another example of an immunodeficiency that is not associated with cryptococcosis are *MYD88* defects. Myd88 is the main signaling molecule of Toll-like receptors and therefore genetic LOF increases susceptibility to several infections but has not been associated with development of cryptococcosis (Lanternier et al., 2013; Picard, Casanova, & Puel, 2011).

3.3.2 Cytokines

To gain insights on the chemokine factors required to defend against *Cryptococcus*, several studies focused on determinants of mortality or onset

of cryptococcosis in HIV+ patients, the majority with limited access to ART. In HIV+ patients, TNFα and Il-10 levels were increased in those with disseminated infection (Jarvis et al., 2014, 2013, 2015; Lortholary et al., 1999). There is an immune signature associated with early mortality, characterized by monocyte deactivation (reduced HLA-DR expression and TNFα production in response to lipopolysaccharide); increased serum IL-6, CXCL10, and IL-10 levels; increased neutrophil counts; and decreased T-helper cell type 1 responses (Scriven et al., 2016). This signature predicts mortality in these patient populations, but there is still a lot more to be uncovered to fully explain the onset and/or susceptibility to cryptococcosis.

Any event leading to decrease of IFNγ-dependent immunity, including genetic deficiencies in the interferon response pathway, as well as autoantibodies blocking IFNγ, will increase susceptibility to cryptococcosis. Genetic deficiencies in the interferon pathway cause a syndrome known as Mendelian susceptibility to mycobacterial diseases, since the most common infection in these patients are weakly virulent mycobacteria, but cryptococcal disease is also observed (Bustamante et al., 2014; Chi et al., 2013, p. 02). Autoantibodies to IFNγ are specifically associated with HLA class II molecules HLA-DRB1*15:02–HLA-DQB1*05:01 and HLA-DRB1*16:02–HLA-DQB1*05:02 (Ku et al., 2016; Lin et al., 2016). In patients with low circulating IFNγ, administration of IFNγ is beneficial. In contrast, widespread usage of IFNγ in HIV+ patients was tested but studies found that this therapy did not improve overall mortality (see above) (Jarvis et al., 2012). In mouse models, sterilizing immunity is critically dependent on the IFNγ-STAT1 axis, and particularly macrophage iNOS (Leopold Wager et al., 2014; van Dyke et al., 2017). The H99-γ strain of *C. neoformans* (which expresses IFNγ) is an effective vaccine (Wormley et al., 2007). Therefore IFNγ, due to its central role in triggering effective immune responses in macrophage monocytic population, is critically required to resist infection by *Cryptococcus*.

In mice there is evidence that type I interferons (IFNα, β) are also required for defense against cryptococcal infection since type I interferon-deleted mice are more susceptible to infection with *C. neoformans* than wild-type mice (Biondo et al., 2008). There is so far no confirmation of the role of type I interferons in human patients. A recent study found that mice were protected by administration of polyinosinic-polycytidylic acid (polyIC), a bacterial RNA mimetic which induces type I interferon responses. This study found difference in immune requirements required to mediate polyIC protection to *C. gattii* vs *C. neoformans*: depletion of CD4-T cells with DTR showed CD4 T-cells were dispensable for polyIC

resistance to *C. gattii* but critical for polyIC resistance to *C. neoformans* (Davis et al., 2019). In this model, CD8 T-cells were apparently critical for resistance against *C. gattii* challenge. Further confirmation of this study should be performed as it suggests an immune difference dependent on fungal species. Akin to H99γ strain from Wormley laboratory, engineering a cryptococcal strain to produce mammalian TNFα was beneficial to the mouse host but this strain did not induce sterilizing immunity (Fa et al., 2019), suggesting a lesser role of TNFα when compared to IFNγ.

3.3.3 Cellular immunity and T-cells

T-cells are considered the organizers of immune response because specific subpopulations of T-cells have a role in shaping the immune response allowing the tailoring of the immune response to best neutralize the microbial invader. Of all the subsets of T-cells, the most critical for defense against cryptococcosis are CD4+ T-cells, albeit CD8+ T-cells play a supportive role. Disseminated and often fatal infections by *C. neoformans* are the most frequent infections (together with human papillomavirus) in patients with defects in CD4+ T-cell function or numbers (Netea et al., 2004; Panackal et al., 2017; Thornton, Larios, Grossman, Griener, & Vaughan, 2019; Yarmohammadi & Cunningham-Rundles, 2017). In a child with idiopathic CD4 T-cell loss, IL-2 therapy was beneficial as it increased T-cell numbers and presumably their function (Yilmaz-Demirdag et al., 2008). In cases of lymphopenia, recombinant IFNγ is used successfully to aid antifungal therapy (Netea et al., 2004). In HIV+ patients without ART and with CD4 T-cell count ≤ 100 cells/μL (or even ≤ 200 cells/μL), it is recommended that the patients be tested for cryptococcal antigenemia (Ford et al., 2018; Jarvis et al., 2014). In HIV+ patients showing CD4 counts ≤ 100 cells/μL, the stratification of circulating CD4 T-cell counts (<25, 25–49, 50–99) does not predict overall mortality or fungal burden in an early study (Jarvis et al., 2014), and in a recent study CD4 counts >100 cells/μL was weakly associated with decreased mortality (Tugume et al., 2019). HIV+ patients with higher proportion of cryptococcal-antigen specific, TNFα- and IFNγ-producing memory CD4 T-cells showed lower fungal burdens and 2-week mortality (Jarvis et al., 2013), demonstrating that the correct activation of T-cells is required for defense against this fungal pathogen. Mouse models recapitulate this critical role of CD4 T-cells (Aguirre, Crowe, Haas, & Smith, 2004). Effective responses of T-cells are associated with Th1 activation, aimed at killing *Cryptococcus*. The Th1 activation is balanced by a small contribution of Th2 cells to prevent excessive tissue

damage (Mukaremera & Nielsen, 2017; Wiesner et al., 2015). Curiously, IL-17 and Th17 responses, the third subset of T-helper cell's responses, are associated with chronic or invasive candidiasis but thus far not associated with onset of cryptococcosis in humans (Okada, Puel, Casanova, & Kobayashi, 2016), albeit they seem to be beneficial in mouse models (Müller et al., 2007).

3.3.4 Antibody-mediated immunity and B-cells

B-cells are classically known as the producer of antibodies and central to antibody-mediated (or humoral) immunity, though B-cells are also very efficient antigen-presenting cells. Produced in high amounts, antibodies binding to their microbial target neutralize microbial invaders by inhibiting action of toxins and increasing clearance of the microbial threat. In addition antibodies may have direct antimicrobial activity and immunomodulatory roles. Antibody-mediated immunity and B-cells are beneficial for defense against cryptococcosis (reviewed in Casadevall & Pirofski, 2012; Rohatgi et al., 2017). In fact, HIV infection affects B-cell function, resulting in hypergammaglobulinemia (Berberian, Shukla, Jefferis, & Braun, 1994; Kardava & Moir, 2019). In addition, B-cell and antibody responses have been implicated in the onset of IRIS, as there is significant association between lower circulating levels of carbohydrate-specific IgM and susceptibility to IRIS in humans (Yoon, Nakouzi, et al., 2019). Low levels of anti-GXM IgM are correlated with risk to develop cryptococcosis in transplant and HIV patients (Jalali, Ng, Singh, & Pirofski, 2006; Subramaniam et al., 2009; Yoon, Nakouzi, et al., 2019), while higher levels of IgG are correlated with a higher risk for HIV-associated cryptococcal disease (Rohatgi et al., 2017). The important role of antibody-mediated immunity is also demonstrated by association of variants in Fc-receptors with susceptibility to cryptococcosis. In the HIV-positive population, only *FCGR3A* was identified as risk factor to cryptococcosis (Rohatgi et al., 2013). Specific polymorphisms in *FCGR2A* and *FCGR3A* have been associated with risk for cryptococcosis in HIV-negative populations (Hu et al., 2012; Meletiadis et al., 2007; Rohatgi et al., 2013, 2017). The reason for this distinction in susceptibility between HIV-positive and HIV-negative populations is still unclear. The beneficial role of B-cells was further confirmed in a model of $Rag^{-/-}$ mice which lack T- and B-cells, and which showed the injection of naïve B-cells showed B-cells mediate at least a partial protection to cryptococcosis (Dufaud, Rivera, Rohatgi, & Pirofski, 2018). Mice deleted for B-cells die quicker in response to intravenous

and intratracheal infections (Rivera, Zaragoza, & Casadevall, 2005). Finally, deficiency in IgM was protective in mouse models in intraperitoneal cryptococcosis (Subramaniam et al., 2010), underscoring a tissue specific action of IgM.

3.3.5 Mononuclear phagocytes and dendritic cells

The monocytic mononuclear system is a highly plastic (Mosser & Edwards, 2008) and dynamic subset of immune cells. Due to their role as resident cells in every tissue, these are the first immune cells to contact and respond with microbial invaders. These immune cells can also interface with adaptive immunity as they are efficient antigen presenters, and act as effector cells, as they can be activated and directed to perform antimicrobial functions and upon resolution of inflammation they direct tissue repair (Mosser & Edwards, 2008). Gene variants and autoantibodies leading to decrease function of macrophages are frequently associated with cryptococcal disease. Autoantibodies against granulocyte-macrophage colony-stimulating factor macrophage (GM-CSF) will affect monocyte and granulocyte numbers and function and are also frequently diagnosed in association with disseminated cryptococcosis (Applen Clancey et al., 2019; Crum-Cianflone et al., 2017; Kuo et al., 2017; Rosen et al., 2013; Saijo et al., 2014). The majority of cytokine deficiencies which affect immunity to *Cryptococcus* have major effects in macrophage function, when compared to other immune populations. This is confirmed by mouse models where monocytic-macrophage specific deletions (using the lysozyme promoter) showed that deletion of *Stat1* in this population recapitulates the immune defects in fighting cryptococcal infection (Leopold Wager et al., 2014). It is therefore well established that the tissue-resident macrophages and infiltrating monocytes play a critical role in defending against *Cryptococcus* (Coelho et al., 2014).

Depletion of macrophages can also be achieved by injection of clodronate which results in depletion of macrophages in several tissues (Charlier et al., 2009; Sun et al., 2019; Van Rooijen & Sanders, 1994) and this technique demonstrated that depletion of macrophages prior to fungal infection worsens outcomes in mouse models (Sun et al., 2019). In contrast, the injection of clodronate to deplete macrophages after the establishment of infection lead to decreased brain burden of macrophages (Charlier et al., 2009), a paradoxical finding that lead to the positing of the Trojan-horse hypothesis, whereby yeasts disseminate throughout the body while traveling inside macrophages. While it is accepted that in some

conditions macrophages can harbor yeasts and become a safe haven for pathogenic yeasts, overall macrophages are critically required to control and contain cryptococcosis.

Depletion of the monocytic population has shown some discrepant results. *CCR2*$^{-/-}$ mice, which lack inflammatory monocytes (CCR2^{+}), found the inflammatory monocytes to be beneficial for mouse survival (Traynor, Kuziel, Toews, & Huffnagle, 2000). In contrast selective depletion of monocytes before and during the first 3 days of infection, by injecting diphtheria-toxin into mice expressing diphtheria-toxin receptor in the CCR2^{+} population, found that mice depleted of CCR2 inflammatory monocyte population survived longer than wild-type mice (Heung & Hohl, 2019), which suggested a detrimental role of monocytes in the early stages of infection. The cause of this discrepancy is unresolved. Overall, much remains to be elucidated regarding the roles of specific subsets of the monocytic-macrophage populations as well as their mechanisms to kill *Cryptococcus* (Coelho et al., 2014).

3.3.6 Neutrophils

For the majority of fungal pathogens, neutrophils are protective and critically required to prevent disease, but this is not the case for *Cryptococcus*. Human and mouse neutrophils can kill *Cryptococcus* in vitro (Chiller, Farrokhshad, Brummer, & Stevens, 2002; Mambula, Simons, Hastey, Selsted, & Levitz, 2000). In mouse models neutrophils help to clear yeasts cells from brain vasculature (Zhang et al., 2016). However mouse models show neutrophils are mostly associated with detrimental responses. Depletion of neutrophils via Gr1-binding antibody is associated with longer survival and decreased mortality of the infected mice (Mednick, Feldmesser, Rivera, & Casadevall, 2003) demonstrating a detrimental role for neutrophils in this infection, albeit the underlying mechanisms remain unexplored. There is still much to be deciphered in these models, particularly as tools allowing the selective deletion of immune populations, which will allow for a full dissection of these integrated responses, are still being developed.

3.3.7 Other immune cell populations, pattern-recognition receptors and hormones

There are comparatively fewer studies investigating the role of immune populations such as eosinophils, mast cells, and NK cells. For example, NK cells greatly enhance anti-cryptococcal activity of macrophages in vitro (Kawakami et al., 2000) and humans NK cells inhibit fungal

growth in vitro (Levitz, Dupont, & Smail, 1994). Overall the contribution of these cell types to protection against cryptococcosis warrants further investigation.

Mannose-binding lectin deficiencies, caused by gene variants in *MBL2* was found to predispose to cryptococcosis in a Chinese population (Ou et al., 2011) but not in Australian population (Eisen et al., 2008).

There is a sex bias in risk for cryptococcosis, with males having a higher risk for disseminated cryptococcosis (Guess, Rosen, & McClelland, 2018), even after accounting for factors such as increased rates of HIV and possibly higher environmental exposure due to social patterns. The molecular mechanisms underlying the sex bias are still mostly unknown but one study has found an influence of sex hormones in T-cell's functions (Guess, Rosen, Castro-Lopez, Wormley, & McClelland, 2019).

Acknowledgments

C.C. and R.A.F are part of the Medical Research Council Centre for Medical Mycology MR/N006364/2. R.A.F. is supported by a Wellcome Trust Seed Award (215239/Z/19/Z). We thank Nicolas Helmstetter for proofreading.

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